

RECENT ADVANCES IN
CLINICAL PATHOLOGY



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which necessitates several examinations of faeces before a negative report is given. Persistent carriers usually have demonstrable specific antibodies in their blood, *e.g.* the suspected carrier of the typhoid bacillus may be identified as a result of the presence of agglutinins, particularly Vi, in the patient's serum. Patients who have suffered from an acute attack of intestinal infection may continue to excrete the organism in the faeces for varying periods, and sometimes intermittently, so that it may be difficult to decide when the patient is free from infection. The usual demand is for three negative tests taken at not less than three-day intervals; this is a good working rule, but an occasional patient who excretes the organism intermittently may be missed. It should be remembered that though excretion of these organisms is most commonly via the faeces, in the enteric group it may occur via the urine or from any other part of the body which may have become the seat of localised infection.

Food is probably the commonest vehicle of infection. The organism may be introduced to it during handling, or may originate from the same source as the food itself. For example during the present war dried egg powder has been an important source of first-class protein. Poultry are susceptible to *Salmonella* infections, and commonly become healthy carriers of this group of organisms, which may occur in dried egg.

1. The commonest cause of food poisoning is still *Salm. typhi-murium*, of which the natural reservoirs are mice and human carriers. The next commonest cause is *Salm. enteritidis*; this organism has been isolated from rodents, cattle and duck eggs. *Salm. thompson* is the third commonest cause of food poisoning; the natural reservoir of this organism is unknown, though it has been isolated on occasion from pig mesenteric glands, and from chickens.

Food poisoning due to pre-formed toxins, other than those of *Cl. botulinum*, is of relatively frequent occurrence. It differs from bacterial food poisoning in that the incubation period is short, usually between one and eight hours with an average of about four hours. The disease is characterised by severe vomiting and collapse as the predominant symptoms. The food is usually found to be heavily contaminated with *Staphylococcus aureus*, but sometimes with streptococci or aerobic sporing bacilli—in fact, it now seems probable that many organisms, if they are present in sufficient numbers, are capable of producing toxic food poisoning. These toxins seem to be relatively heat-resistant.

ISOLATION OF THE ORGANISM

Faeces: The macroscopic appearance of the faecal specimen may be of value in the tentative differentiation between Salmonel and dysentery infections, as the former usually produce a very watery stool containing some degenerate pus cells, whereas the latter produce a mucoid stool which may contain bright blood and numerous, relatively normal pus cells and macrophages. The best material for culture is a piece of mucus, which may be washed in sterile saline if considered necessary. Where specimens have to be sent through the post it is advisable to mix the faeces with glycerin-saline (see Appendix, 1). The rectal swab is a useful and easy method of collecting the faecal specimen, particularly in outbreaks of dysentery among children in residential institutions. The swab used should be a strong wire, the end of which is roughened to make the cotton wool adhere. It must be passed into the rectum using a speculum of rubber or glass if need be, and cultured as soon as possible after taking.

Semi-solid or fluid faeces or other material may be used directly for the inoculation of all media, but should the material be hard, a thick suspension in broth or peptone water should be used. Primary cultures should be made on fluid (enrichment) and solid (selective) media. The fluid medium is helpful when the organisms are rather scanty as in *Salmonella* infections or in convalescent or contact carriers of the enteric group. A satisfactory fluid medium is Kauffmann's tetrathionate broth (see Appendix, 2), which should receive a heavy inoculum; it should be plated on desoxycholate citrate agar after 24 and 72 hours' incubation. There are numerous solid media in use at the present time; of these desoxycholate citrate agar and Wilson and Blair's bismuth sulphite medium have been found to be most useful (see Appendix, 3 and 4).

Organisms of the enteric, *Salmonella* and dysentery groups in the smooth phase grow freely on desoxycholate citrate agar (Hynes 1942). The following description is taken from Hynes' paper:

"The medium does not deteriorate after being poured. The less strains of *B. coli* which grow on it give pink umblicated colonies 2-2 mm. in diameter and cause (by acid production) an intense precipitation of desoxycholate in the surrounding medium. *Aerogenic* colonies are domed and mucoid. All *coli* colonies are completely opaque. The colonies of pathogens by contrast are colourless and relatively translucent, and by their alkaline reaction they redissolve the desoxycholate, so that they are surrounded by a zone of transparent

medium. The colony of *Sh. sonnei* grows from 1 mm diameter after 18 hours' incubation to 2 mm after 38 hours, it may be colourless on the first day, but by the second it is usually a characteristic pale pink, much paler and less opaque than *B. coli*. The colony of *Sh. flexneri* is colourless, often with a narrow plane periphery round a central dome. Colonies of *Salm. paratyphosum B* grow from 1 mm. diameter after 18 hours' incubation to 2-4 mm on the second day. Other *Salmonella* organisms are similar. After 1 day's incubation the colony of *Salm. typhosum* is $\frac{1}{4}$ -1 mm. in diameter and pale pink, a day later it is flat, conical, 2 mm in diameter, colourless and slightly opaque. It may have a central pale grey dot. Of the non-pathogenic non-lactose fermenters, only *Proteus* strains grow freely. Their colonies may often be recognised by their translucency and refractility, but they can closely simulate any of the pathogens. Many have a large central black dot, and most have a characteristic fishy smell."

Wilson and Blair's medium is particularly useful for the isolation of *Salm. typhosum* and *Salm. paratyphosum* but is less reliable for the isolation of other members of the *Salmonella* group and is useless for the dysentery group. Poured plates of the medium should ordinarily be used within 24 hours of preparation and the surface must be dry; plates are useless once they have a greenish colour. After 24-48 hours' incubation, *Salm. typhosum* and *Salm. paratyphosum B*. appear as jet black colonies which may be surrounded by a dark zone with a metallic sheen. Other members of the *Salmonella* group appear as brown, black or dark green, flat colonies. Occasionally some non-pathogenic members of the *Bacterium* group will grow, but these tend to be rather large (4 mm. in diameter), dome-shaped and shiny, some being mucoid; their colour varies from pale green, dark green to black.

There are other media in use in different laboratories, such as eosin-methylene blue, etc., but there is no evidence that they are in any way superior to, and it is doubtful if they are as useful as, the media mentioned.

Blood, urine etc. The investigation of a case of enteritis must always aim at the isolation of the infecting organism at the earliest possible moment. In enteric (typhoid and paratyphoid fever) which are systemic or generalised infections, this may necessitate the taking of blood cultures or the culture of blood clot from the patient. Blood (in the proportion of 1:10-1:15) may be added directly to plain or glucose broth; blood-clot should be broken up and added to bile-broth. Vomit and urine may also be cultured in tetrathionate broth and on desoxycholate citrate agar.

The investigation of soft or fluid foods for the presence of

Salmonella organisms may follow the routine for faeces. Where meat, for example a ham bone, is suspected, material should be taken from different depths in the meat and also the marrow from any bones present. The meat may be ground with sterile sand and broth, or else finely chopped with sterile scissors and the usual media inoculated.

IDENTIFICATION OF THE SALMONELLA GROUP

Biochemical: The full identification of organisms of the Salmonella group must include the biochemical reactions as well as the serological reactions. The biochemical reactions to be tested are those with lactose, glucose, mannitol, sucrose, salicin, litmus milk and for production of indole. Other useful tests are fermentation of dulcitol and liquefaction of gelatin. Members of the Salmonella group pathogenic to man fail to ferment sucrose and salicin, do not produce indole (except some strains of *Salm. eastbourne*), and do not liquefy gelatin (except *Salm. dar-es-salaam*, *Salm. abortus-bovis* and *Salm. schleissheim*).

Slide agglutination. The preliminary serological identification may be done by the slide agglutination technique. The suspected smooth colony, or growth from an agar slope is picked off with a stab wire and rubbed up in a large loopful of known agglutinating serum on a slide. Examine the mixture with a dissecting microscope using oblique illumination, or against a black background. Agglutination appears within a few seconds of mixing. A row of different sera can be arranged on a slide and suspensions made in the sera without flaming the wire in between as the amount of serum carried over is negligible.

Having determined that the culture is motile, test first with polyvalent salmonella "H" (flagellar) antiserum¹; a positive reaction indicates that the organism is probably a member of this group. Next test with "O" (somatic) sera in order to determine the somatic antigenic group. Then consult the Kauffman-White table and where (as in the IV or *Salm. paratyphosum B* "O" group) many members have a non-specific "H" phase, test for this. If no agglutination

¹ This serum contains specific "H" agglutinins for all known members of the Salmonella group, and also for the non-specific phase of all diphasic members of this group. Agglutination with this serum indicates that the organism is a member of the Salmonella group. Final identification must depend on the range of the sera available. This and other anti-sera are made available by the Standards Laboratory for Serological Reagents.

is obtained with non-specific *Salmonella* "H" serum, test for specific "H" agglutination.

Example : Motile organism gives agglutination on slide with IV

(*Salm paratyphosum* B "O") sera

Test with non-specific *Salmonella* "H" serum—no agglutination

Test with specific "H" serum "b" (*Salm paratyphosum* B "H" sp)

"i" (*Salm. typhi-murium* "H" sp)

"d" (*Salm. typhosum* "H" sp)

"eh" (*Salm. newport* "H" sp.)

etc.

If agglutination be obtained with IV (*Salm paratyphosum* B "O") and "d" (*Salm. typhosum* "H") then the antigenic structure is IV, d, which is that of *Salm stanley* (see Kauffmann-White table)

Should the organism be in the non-specific "H" phase, it cannot be identified until the specific phase has been isolated, which is often possible by means of the Craigie tube.

The narrow glass tube should have a bevelled lower end, and should project $\frac{1}{2}$ in. above the level of the medium. The medium is 0.3 per cent. nutrient agar to which are added 4-5 drops of undiluted non-specific *Salmonella* "H" anti-serum. Make a stab inoculation into the narrow inner tube and incubate until the organism has grown to the surface of the medium in the outer test tube (usually overnight). Subculture on an agar plate from the surface of the agar in the outer test tube, and incubate. Test a number of colonies by slide agglutination with non-specific "H" serum until one is obtained which fails to agglutinate with this serum. Provided that "H" antigen is present as shown by the motility of the organism, this colony must be in the specific phase and should be used for further tests. Occasionally more than one passage through Craigie tubes may be necessary to induce organisms in the specific phase to grow out from the culture.

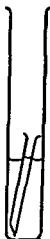


FIG 1

The sera supplied by the Standards Laboratory for Serological Reagents, Colindale, London, should be diluted 1/5 for slide agglutination. A negative test needs no further confirmation; positive results by this method should be confirmed by tube agglutination.

Tube agglutination : Tests should be done with killed suspensions.

For the "O" agglutination test it is essential that only smooth cultures be employed. Wash the growth from an agar slope with 1 cc. saline and transfer the suspension to a clean test tube. Add

about 7 cc. of absolute alcohol and shake up very thoroughly; centrifuge and remove the alcohol; resuspend the deposit in normal saline and add one drop of formalin. This suspension will respond to "O" agglutinin only, and is ready for use after dilution with saline to the required density (300×10^6)

For "H" agglutination, an overnight broth culture to which a few drops of commercial formalin have been added is most satisfactory. Saline should be added until the required density is obtained (300×10^6). Tube agglutination can be carried out by the Dreyer technique or by "double dilution."

Some organisms of the enteric *Salmonella* group have another somatic antigen known as Vi which prevents such organisms from being agglutinated by the usual "O" sera. *Salm. typhosum* commonly occurs in the Vi phase when first isolated and tests for this antigen must be made with the specific Vi antiserum by slide and tube agglutination. The Vi antigen is very labile and tests must be made on living material taken from agar slopes and suspended either in physiological saline or in mercuric iodide solution 1/1,000 (see Appendix 6)

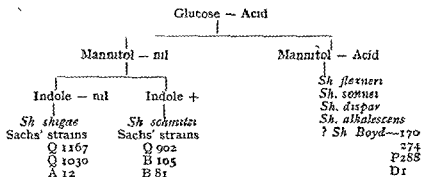
Phage-typing: It is now possible to subdivide the members of a single type of *Salmonella* by phage-typing. This method is used for typing *Salm. typhosum* and is being extended to other members of the *Salmonella* group; so far the work is still in the experimental stage. This method is of special use in epidemiological investigations, particularly in tracing the source and mode of spread of the epidemic (see Craigie and Yen, 1938; Felix, 1943).

Pitfalls: Various difficulties may arise in the identification of any strain. The preliminary identification by slide agglutination is commonly done on material from selective media. Such material may give a slightly granular suspension which with experience can be differentiated from true agglutination. The inhibitory substances in the media sometimes suppress the growth of flagella, thereby making the identification of the "H" antigen impossible. These strains rapidly become motile on subculture.

Some rabbit sera contain naturally occurring α agglutinins against antigens found in some strains of *Proteus* and the para-colon group. These organisms usually give atypical sugar reactions after prolonged incubation. The presence of these α agglutinins in a diagnostic serum may give rise to false positive results, particularly with the slide-agglutination technique. However, these antibodies are not present in appreciable amounts in diagnostic sera, and therefore give no agglutination in the confirmatory tube tests.

IDENTIFICATION OF THE DYSENTERY GROUP

The same reagents should be used for the biochemical reactions of the dysentery group as for the *Salmonella*. The dysentery organisms fail to ferment lactose or sucrose (except some strains of *Bact. sonnei* and Sachs' strains B 105 and B 81) and fail to liquefy gelatin



Most strains of the Flexner type, with the exception of the Newcastle bacillus, produce indole (Boyd, 1940). No strains of *Bact. sonnei* are indole-positive, but recently three strains of this organism which fermented glucose only have been investigated. The biochemical reactions of the Newcastle group are extremely variable and may vary with the type of peptone water base used for the medium. Acid production with a bubble of gas in glucose only is the most common finding in Britain. All strains are indole-negative.

The serological identification of dysentery organisms may be done by slide technique and confirmed by tube agglutination. Final identification of dysentery types of the Flexner group must depend on agglutinin absorption tests or the use of sera from which the group antibodies have been absorbed as these organisms have antigens in common.

Boyd (1940) has shown that the Flexner group of organisms have type antigens specific to each variety and also group antigens of which the whole or part are common to all members of the group. types X and Y described by Andrewes and Inman are organisms containing group antigens only, the specific antigen having been lost. On this finding he suggests that an organism should not be included in the Flexner group unless it contains antigens common to the group.

Wheeler (1944), whose table giving the antigenic analysis of *Sh.*

flexneri is given below, confirmed Boyd's findings. Boyd has also described a number of dysentery bacilli which have caused disease in man but which are unrelated to the Flexner types. These are referred to as ? *Sh. boyd* as the International Nomenclature Committee has not met so far to decide on a name for these organisms.

TABLE I
ANTIGENIC ANALYSIS OF FLEXNER GROUP

Andrewes and Inman and Boyd types	Antigenic Structure		Flexner Type Designation
	Type specific	Group	
V	I	1 2 4 5 6 9 . . .	I
W	II	1 3 4 . . .	IIa
		1 7 8 9 . . .	IIb
X	*	1 7 8 9 . . .	
Y	*	1 3 4 . . .	
Z	III	1 6 7 8 9 . . .	III
103	IV	1 6 . . .	IV
Pir9	V	1 5 7 9 . .	V
88—Newcastle	VI	1 2 4 . . .	VI

* No major type-specific antigen in strains studied.

Reference: K. M. Wheeler. *J. Immunol.* 48. (1944) 99.

ANTIBODIES IN PATIENT'S SERUM

The diagnosis of enteric and *Salmonella* infections may be made on the agglutination reactions of the patient's serum. The type of organism used for the reaction must depend upon the clinical disease of the patient, together with a knowledge of the type of infecting organisms which have occurred in the part of the world from which the patient comes. In Great Britain *Salm. typhosum* and *Salm. dysenteriae* are the common causes of enteric fever, the former may be caused by *Salm. typhosum* and the latter by *Salm. dysenteriae*. In the diagnosis of enteric fever, one or two bacteria antigens which must be included in serological investigations. It has been found that the "O" agglutinins which

develop in human sera may not show the specificity which would be expected from the antigenic structure of the infecting organism. The serum from a patient with paratyphoid B infection may agglutinate *Salm typhosum* "O" to a higher titre than *paratyphosum B* "O", so that the diagnosis of the type of infecting organism from the presence of somatic agglutinins alone is rarely possible. The antibody response to the flagellar antigen is related, as would be expected, to the antigenic structure of the infecting organism. In the case of diphasic organisms, the agglutinins to both or either phase may show a rise, but the rise is rarely the same with both phases and commonly one type of agglutinin only is present. During an epidemic of *paratyphoid B* (I., IV, V, XII, b \longleftrightarrow 1, 2 . . .) infection it is not uncommon to find some patients with a rise in b agglutinins, others with a rise in 1, 2 . . . The rise in somatic agglutinins usually occurs early in the disease and is followed by a rise in the "H" agglutinins, which may persist for long periods after the original infection has been overcome. As a result of the war, a large proportion of the population has received prophylactic inoculations of typhoid, paratyphoid A and B bacilli and possibly paratyphoid C, which makes the interpretation of results more difficult as the agglutinins to these organisms may persist and may rise as the result of any febrile disease. The diagnosis of enteric infection on the agglutination reaction is based on the level of specific agglutinins reached in the patient's serum and expressed as titres, that is the final dilution at which agglutination is observed. The normal un inoculated population may show the presence of natural "H" agglutinins to the enteric group of organisms to a titre of 1/50, but rarely show "O" agglutinins. During the course of disease the titres to the infecting organism rise, reaching a maximum level towards the end of the second week of the disease. The diagnosis of enteric infection therefore involves the use of "O" and "H" suspensions, the usual preliminary test in this country being with *Salm. typhosum* "H" and "O", *Salm. paratyphosum B* "H" and "O" and non-specific *Salmonella* "H" (1, 2 . . . 1, 5 . . .). A titre of 1:100-1:200 with "O" antigen or 1:500-1:1,000 with "H" antigen is suggestive of infection. Where possible the patient's serum should be investigated during the first week of the disease and again after an interval of not less than five days; if there is a marked rise of titre on the second test, then the diagnosis is fairly definite. Where possible, the double test should always be carried out on patients who have received T.A.B. vaccine or who have a history of previous enteric infection.

The presence of Vi antigen in *Salm. typhosum* has already been mentioned. Agglutinins to this factor may rise during infection about the 16th to the 20th day of the disease; the rise occurs earlier in patients who have had T.A.B. inoculations. A titre of 1/10 may be regarded as suggestive of infection. The Vi agglutination is of great value in the diagnosis of the carrier state, and here again a titre of 1/10 is suggestive. Freshly drawn sera may have an inhibitory effect on the reaction, so all sera should be inactivated by heating to 45° C. for half an hour. The test should be carried out in test tubes 2 in. by 0.5 in., using 1 cc. amounts of the diluted serum and starting at a dilution of 1/5. The suspension used for the test is made from an organism which will react with Vi agglutinins only, and is unaffected by agglutinins to *Bact. typhosum* "H" and "O". The density of the suspension is equivalent to 4,500 million *Bact. coli* per cc. 0.05 cc. of suspension is added to each tube. The tubes are shaken, incubated at 37° C. for 2 hours, then left at room temperature for 22 hours. To read the reaction the tubes should be lifted carefully from the rack, care being taken not to shake them. A positive reaction shows a granular opacity of the supernatant fluid and a deposit which covers the whole of the bottom of the tube. The control tube shows a clear or even suspension in the supernatant fluid with an opaque deposit about 4 mm. in diameter in the centre of the bottom of the tube.

If different total amounts of fluid are used per tube, the opacity of the suspension must be altered proportionately as the test is dependent upon the actual number of organisms present which can react with the serum.

Specific antibody production is less constant in Salmonella food poisoning infections and in the dysenteries than in enteric fever. Search for antibody in such cases may be made in order to establish the diagnosis retrospectively. Although the specificity of the reaction with the dysentery antigens is in doubt since titres of 1:100 or over to the Flexner group of dysentery organisms may be found in the blood of normal adults, a titre of 1:20 to *Sh. sonnei* in the blood of a child is suggestive of infection with that organism.

The serum used for agglutination tests should not show haemolysis. If it is necessary to use such a serum, the reactions should be incubated at 37° C. overnight and read the following day.

Suspensions used for diagnostic work must be of a constant agglutinability; therefore it is necessary to obtain them from a reliable source, such as the Standards Laboratory for Serological Reagents, Colindale, London, N.W. 9.

Appendix

(1) Sachs' Solution:

Glycerol	1,000 cc.
0.85 per cent sodium chloride	2,000 cc
Saturated aqueous solution of phenol red and 5 per cent solution of Na_2HPO_4	

Add the 2,000 cc saline to the 1,000 cc. glycerol. Add sufficient phenol red and Na_2HPO_4 to give a reaction of pH 8.0, the two being added separately. Add 5 or 10 cc. to 1 oz. screw-capped bottles. Sterilize for 10 minutes at 10 lb pressure. The reaction after sterilization should be pH 7.4

(2) Tetrathionate Medium.

1. Sterile nutrient broth, pH 7.4 900 cc.
2. Sterile chalk 50 g.
3. Sodium thiosulphate 50 g, dissolved in water, made up to 100 cc and steamed for 30 minutes
4. Iodine 5 g and potassium iodide 4 g, ground up in a mortar and dissolved in 20 cc of distilled water

These four constituents are kept already sterilized. For use, these ingredients should be mixed under strictly aseptic conditions, and distributed in 10 cc quantities into sterile tubes. No sterilization of the finished medium is required.

Kauffmann's Modification.

To a flask containing 500 cc of tetrathionate broth made according to the original formula, add —

- (1) 5 cc of a 1/1,000 Brilliant Green Solution (final concentration 1/100,000)
- (2) 25 cc sterile ox bile—making a concentration of 5 per cent

Shake well to distribute the calcium carbonate, tube in suitable amounts, then steam for 30 minutes.

Kauffmann states that this broth keeps well.

(3) Desoxycholate-citrate Agar. For the isolation of Dysentery, Paratyphoid and Salmonella Organisms.

Agar.	22.5 g.
Lab. Lemco	5.0 g.
Difco proteose peptone (or Evans)	5.0 g
Lactose	10.0 g.
Sodium citrate	8.5 g.
Sodium thiosulphate	8.5 g.
Ferric citrate	1.0 g
Sodium desoxycholate	5.0 g
Neutral red (as indicator)	
Water to 1,000 cc.	

Dissolve 20 g. Lab. Lemco in 200 cc. water over the flame; make just alkaline to phenolphthalein with 50 per cent. NaOH, boil and filter. Adjust the pH to 7.3, make up the volume to 200 cc. and add 20 g. Difco proteose peptone.

Dissolve 90 g. agar in 3,700 cc. distilled water by one hour's steaming. Filter the agar, add the Lab. Lemco-peptone solution and mix.

Add 5 cc. 2 per cent. neutral red and 40 g. lactose, and mix.

Bottle in accurate 100 cc. lots and sterilize by free steam (and up to 5 lb.) for 1 hour.

SOLUTION A:

Sodium citrate (A.R., $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) 17 g.

Sodium thiosulphate (A.R., $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) 17 g.

Ferric citrate (scales) 2 g.

Distilled water 100 cc

Dissolve by heat or by standing at room temperature for 2 days

SOLUTION B.

Sodium desoxycholate 10 g.

Distilled water 100 cc.

These solutions need *not* be sterilised.

FOR USE melt 100 cc of the agar base, and add 5 cc. each of solutions A and B in this order, using separate pipettes and mixing well between. Pour plates *immediately* and dry the surface.

REFERENCE. LEIFSON, E. *J. Path. Bact.* 40. (1935) 581.

NOTES ON THE USE OF DESOXYCHOLATE-CITRATE AGAR

1. The type of protein extract and peptone used greatly affects the performance of the medium, and the products recommended should not be varied without control experiments to ensure that the performance of the medium is not impaired.

2. The medium should be poured and cooled as soon as possible after the addition of the desoxycholate, otherwise it tends to become very soft.

3. It is no disadvantage if a rather acid reaction of the medium causes partial precipitation of the desoxycholate. Simply rubbing a loop on the medium may cause such precipitation along its track giving a false appearance of contamination.

4. The desoxycholate must be pure, as all the common impurities impair the efficiency of the medium.

The medium is pale and slightly opaque. Some coliform strains and particularly *Bact. aerogenes* grow on it, producing deep pink opaque colonies 1-2 mm. in diameter, and causing (by acid production) an intense precipitation of desoxycholate in the surrounding medium.

The colonies of pathogens are colourless, and by their alkaline reaction redissolve the desoxycholate, so that they are surrounded by a zone of transparent medium.

Sh. sonnei is round, about 2 mm in diameter, with a well-defined edge and no appearance of "roughness". Rough variants of this organism do not grow on the D.C. medium. The colonies may be pale pink or become so on further incubation or storage. *Sh. flexneri* is similar, but may have a narrow plane periphery surrounding a central dome. Colonies of *Salm. paratyphosum B* and *Salmonella* are larger, 1-4 mm in diameter, often with a central black dot after 48 hours' incubation. *Salm. typhosum* gives a flat, round colony.

Of non-pathogenic non-lactose fermenters, only *Proteus* strains grow freely; the colony is usually glassy and more translucent than those of the pathogens: most strains have a central black dot. There is no tendency to spread, but the characteristic fishy odour is present.

TECHNIQUE.

Inoculate plates heavily with faeces or rectal swabs in a way that will ensure discrete colonies. incubate for 18-24 hours. Re-incubation for another 24 hours is occasionally necessary if there are no non-lactose fermenting colonies present after 24 hours' incubation, or if the colonies are very small.

Slide agglutination with colonies picked directly from the plate is satisfactory provided the usual precautions are taken to obtain a fairly heavy and uniform suspension.

For fermentation reactions colonies are picked, preferably with a straight wire, into peptone water and subcultured into the appropriate sugars after 4-6 hours' incubation. At the same time, a subculture should be plated on MacConkey's medium to test the purity of the peptone-water culture.

This medium is particularly suited for the isolation of the dysentery organisms prevalent in this country, for the *Salmonella* food-poisoning group, and for *Salm. paratyphosum B*. It is not so selective for *Salm. typhosum* though superior to MacConkey's medium.

(4) Wilson and Blair's Bismuth Sulphite Medium (for the isolation of *Salm. Typhosum* and *Salm. Paratyphosum*).

Stock Bismuth-Sulphite-Glucose-Phosphate Mixture.

Prepare as follows:

Dissolve 6 g. bismuth-ammonio-citrate scales in 50 cc. boiling distilled water. Add to this a solution obtained by boiling 20 g. anhydrous sodium sulphite in 100 cc. distilled water, and then while the mixture is boiling add 10 g. anhydrous sodium phosphate (Na_2HPO_4). To the bismuth-sulphite-phosphate mixture when cool add a solution of glucose obtained by dissolving 10 g. of commercial glucose in 50 cc. boiling distilled water. This mixture will keep for months.

Iron-Citrate-Brilliant-Green Mixture.

The Mixture consists of :

1 per cent solution of iron citrate in distilled water	200 cc
1 per cent. brilliant green in distilled water	25 cc.

This mixture will keep for months.

Make up the medium as follows :

Nutrient agar, 3 per cent (hot, melted)	100 cc.
Stock bismuth-sulphite-glucose-phosphate mixture	20 cc.
Iron-citrate-brilliant-green mixture	4.5 cc.

Pour into Petri dishes Use as freshly as possible. Plates must have a perfectly dry surface before inoculation. Discard if colour is greenish.

The use of this medium depends on the property of *Salm. typhosum* to reduce the sulphite to sulphide in the presence of glucose; and the inhibition of *Bact. coli* by bismuth sulphite in the presence of an excess of sodium sulphite. Isolated colonies of *Salm typhosum* and *Salm. paratyphosum* are black, the former usually appearing within 24 hours and the latter within 48 hours

(5) Eosin Methylene-blue Agar (Levine's Modification) 1918.

Peptone	10 g.
Dipotassium hydrogen phosphate (anhydrous)	2 g.
Agar	15 g.
Lactose	10 g.
Eosin yellow 2 per cent. aqueous solution	20 cc.
Methylene blue 0.5 per cent solution	20 cc.
Distilled water	1,000 cc.

Dissolve the peptone, dipotassium hydrogen phosphate, and agar in water by boiling Make up the loss due to evaporation Sterilize at 15 lb. for 15 minutes

Before use, to each 100 cc. of sterile agar add 1.0 g. of sterile lactose, 2 cc of 2 per cent aqueous solution of eosin yellow, and 2 cc. of 0.5 per cent solution of aqueous methylene blue. No adjustment of reaction nor filtration is necessary The dyes should be weighed only on an analytical balance and added in the order indicated.

(6) Solution for Suspension of Enteric or Dysentery Organisms for Agglutination Tests.

Stock Mercuric Iodide Solution (S.M.I.S.).

Mercuric Iodide	1 g.
Potassium Iodide	4 g.
Distilled Water	100 cc.

ENTERIC INFECTIONS

17

Buffered Formol Saline (B.F.S.).

Two per cent formalin in physiological saline brought to a pH of 7.6 by the addition of Na_2HPO_4

Solution for Use.

S M I S

B F S.

Physiological Saline

10 cc.

2.5 cc.

90 cc.

JOAN TAYLOR

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CHAPTER II

LABORATORY DIAGNOSIS OF TYPHUS FEVER

Serology

THE serology of typhus fever has in recent years been intensively studied. The time-honoured Weil-Felix reaction is still the routine laboratory test for the diagnosis of typhus. The techniques and interpretation of the test have recently been again described by Felix (1942, 1944). There has also been a renewal of interest in the rapid bedside *Proteus* agglutinin test first described by Castaneda.

Recent developments in the cultivation of rickettsiae and the preparation of pure suspensions have stimulated the more general use of rickettsial antigens in the study of typhus. Particular attention has been paid to complement-fixation and rickettsial agglutination tests. The advantage of these methods is in distinguishing between epidemic and murine varieties of the disease.

✓ **Weil-Felix Reaction :** Felix's (1942, 1944) technique, and the classical one of Dreyer both give satisfactory results. It must be remembered, however, that the titres obtained with the same serum by the two methods may be different. The method of Felix usually gives higher readings. One technique should, therefore, be adhered to. This is especially important when diagnosis is based on an increasing agglutinating titre during the course of the illness.

During recent years standardized alcohol-treated suspensions of *Proteus* OX19 have been available from the Standards Laboratory, Colindale, London, for the Weil-Felix test. These are usually entirely satisfactory. In the Dreyer technique, commonly employed, serial dilutions of serum from 1 : 20 are made in conical tubes of approximately 1.0 cc. capacity. Volumes can conveniently be measured by standard drops as in the classical Dreyer's method, and an appropriate range of dilution made. With sera of unknown titre, a wide range of dilutions is often desirable and it may then be convenient to make twofold dilutions using volumetric pipettes and a separate row of dilution tubes. This method is especially convenient when the same serum is to be tested against a number of antigens. After addition of the *Proteus* OX suspension the tubes are incubated at 50-52° C. for two to four hours. It is convenient to leave the tubes at room temperature overnight and to take a final reading after

18 hours. It is advisable to test a serum of known titre with each batch of unknown sera.

With this technique, titres of 1/100 are occasionally encountered in normal individuals. A single reading of 1/200 or more can usually be regarded as significant, but lower titres can be diagnostic if previous sera from the same patient have been negative or have shown agglutinins in lower titre.

In epidemic typhus a diagnostic titre (1/200 or more) is encountered in about 70 per cent. of patients by the end of the first week of the disease. A serological diagnosis can sometimes be made during the first five days by observing a significant rise in the OX19 agglutination titres even if they do not reach 1/200. The serological response is less marked in mild cases of endemic murine typhus and sometimes in grave cases of the epidemic infection.

"Castaneda" Slide Agglutination Test: This test depends on the agglutination of a heavy suspension of *Proteus* OX19 by whole blood on a slide. Staining of the suspension with methylene blue makes the test easier to read. The suspension can be readily prepared by washing off the growth of *Proteus* OX19 from a solid medium, precipitating with alcohol, washing and resuspending in buffered formol saline. Sufficient methylene blue is then added until the suspension has a deep blue colour. The concentration of bacteria should be approximately 18×10^9 per cc.

For the actual test at the bedside, a drop of the patient's blood is taken on a clean glass slide and mixed with a drop of the proteus suspension by repeated tilting. A positive reaction is shown by the formation of clumps (blue, if methylene blue is used) in the form of a ring near the outer margin of the drop. The test cannot replace the Weil-Felix reaction, but has value in field work.

✓ **Rickettsial Complement-Fixation:** Satisfactory antigens have been prepared from yolk sacs of chick embryos and mouse or rat lungs infected with murine and epidemic typhus rickettsiae. The ground-up yolk sacs or mouse lungs in suspension can be used in a relatively crude form, but more highly purified antigens are preferable. Purification of the yolk sac antigens by ether treatment has been described by Craigie (1945). Mouse or rat lung antigens are best purified by differential centrifugalization and kieselguhr treatment (*vide infra*). Usual techniques for the complement-fixation test can be employed.

Rickettsial Agglutination: Suspensions of rickettsiae can be prepared as for complement-fixing antigens. The preparation of

suspensions from the lungs of small rodents is not without risk and accidental typhus infections have occurred amongst laboratory workers using this technique.

Rickettsial suspensions suitable for agglutination tests have been prepared from the lungs of rats or mice infected intravenously with the murine or epidemic typhus rickettsiae (Fulton and Begg, 1946). Consolidated lungs from rats or mice infected with typhus rickettsiae are emulsified in serum broth, and coarse tissue debris is separated by centrifugalization at a low speed. The rickettsiae are deposited from the supernatant by centrifugalization in an angle centrifuge at 4,000 r.p.m. for one hour and resuspended in buffered saline (pH 7.0). A suspension of kieselguhr (Celite F.) is then added. The lung proteins are absorbed and precipitated by the kieselguhr leaving a pure suspension of rickettsiae.

Suspensions from the yolk sac are best prepared by centrifuging the sacs in buffered formol saline (pH 7.0) and then shaking up with ether. The aqueous phase gives a relatively pure suspension of rickettsiae suitable for agglutination tests (see Craigie, 1945).

Agglutination tests are conveniently performed by the technique employed in the Weil-Felix test. Agglutination proceeds more slowly, and care must be taken that the heat-sensitive specific antigens are not destroyed by overheating in the water bath; incubation for 12-18 hours at 37-40° C. is preferable. With pure suspensions of rickettsiae agglutination is easily seen with the naked eye.

Rickettsial agglutinins develop at approximately the same stage of the disease as OX19 agglutinins, or slightly earlier. They allow differentiation between the murine and epidemic varieties which are not differentiated by the Weil-Felix test. Investigation has so far shown no antigenic difference between epidemic typhus rickettsiae from widely separated parts of the world.

Freshly prepared antigens are desirable, in both complement-fixation and agglutination tests, for the differentiation between murine and epidemic typhus. The specific antigens are modified both by heating and prolonged storage; with old suspensions the difference between murine and epidemic infection may become undetectable.

Isolation of Causative Rickettsiae: To isolate strains inoculate whole blood (first week of the disease) or ground-up blood clot (2nd week and later) intraperitoneally into guinea-pigs. There are reports of the isolation of rickettsiae from sternal bone marrow inoculated into chick embryo or mice (intranasally); this needs substantiation.

Adaptation to guinea-pigs by repeated passage (brain to peritoneum) succeeds readily. When once infection has been established in guinea-pigs it is relatively easy to infect mice intranasally, or embryo yolk sac with an inoculum of guinea-pig brain. Adaption to mouse lungs is rapid and it has been possible during a recent epidemic to make use of strains isolated during that epidemic in rickettsial agglutination tests.

Difficulty is experienced in isolating strains from the peripheral blood of patients who previous to infection have received typhus vaccine, even when the blood samples are taken during the early stages of the illness.

Blood Changes

Haematology : Alterations in the blood picture have been frequently and variously described. Conflicting statements are explained by the fact that the blood picture of typhus may vary from case to case in the same epidemic and at different stages of the infection in the same individual .

Marked leucocytosis is occasionally seen in severe uncomplicated typhus, but more usually it is an indication of a suppurative complication. There is almost invariably a polymorph lobe shift to the left during the early stages of the disease, even in the absence of leucocytosis.

A relative or absolute mononucleosis is often encountered. Lymphocytosis occurs during convalescence, often to a very marked degree. Abnormal cells of the lymphocyte or monocyte series are seen in the majority of cases in severe epidemic typhus. They are by no means present at every blood examination, but occur in small numbers in most cases during some stage of the disease.

Chemistry : The increase of blood urea during severe typhus has long been recognised ; the blood urea level is of great prognostic value. Urea retention occurs when there is also oliguria, a reduction of plasma chlorides, and alteration of the blood CO_2 content, and all are associated with functional deficiency of the kidneys. It is probable that the impairment of renal function is due to a combination of renal and extrarenal factors ; among the extrarenal may be the fall of blood pressure and coincident reduction in peripheral circulation frequently present in severe typhus

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CHAPTER III

LABORATORY DIAGNOSIS OF SORE THROAT

THE clinical pathologist investigating "sore throat" will have in mind infection with the diphtheria bacillus, haemolytic streptococcus, Vincent's organisms (fusiform bacilli and spirilla)—less probably, quinsy, glandular fever (infectious mononucleosis) the leukaemias and purpuras, granulocytopenia and syphilis. Rarely the chief pathogen present appears to be *Staphylococcus aureus* or the pneumococcus. A considerable number of attacks do not conform to any of these diagnoses and are generally called "pharyngitis" or "catarrhal tonsillitis"; some of these infections may be attributable to a virus. At times this proportion may be high. The American Commission on Respiratory Diseases (1944) studied 116 soldiers with moderate pyrexia and sore throat with local exudate. Twenty-five per cent. yielded profuse growth of beta haemolytic streptococci and showed a material rise in anti-streptolysin titre with an average leucocytosis of 16,000; these were considered true infections. Another 25 per cent. gave sparse colonies of haemolytic streptococci, showed no significant rise in anti-streptolysin titre and had an average leucocyte count of 9,000. These, with the remaining 50 per cent. were regarded as not being streptococcal infections. A single negative swab is not conclusive, for in this investigation a further 24 per cent. yielded positive plates on the second or third swabbing a few days later.

The frequency of occurrence of these various types of sore throat in the work of a given laboratory naturally varies much with time and place. Of over one thousand patients in one series in London (Begg, 1943) sent into hospital notified as diphtheria, around 60 per cent. proved to be cases of true clinical diphtheria; 5 per cent. were healthy carriers of diphtheria bacilli; about 20 per cent. had tonsillitis; other diagnoses were Vincent's angina, glandular fever, another 1 per cent. haemolytic streptococci were isolated from only 40 per cent. of that group.

Routine Examination of Swabs

Inexperienced juniors and nurses do not always realise the necessity of a clear view of the whole of the fauces and the posterior wall

of the throat. A spatula, hand torch or bed lamp and a swab make poor provision for taking a satisfactory swab if the patient is troublesome or the throat very irritable. A head mirror or self-illuminating spatula or some such combination makes it easy to apply the swab to the affected area, and by gentle rotation, to detach some of the membrane. Nor do nurses always realise how gargling or mouth-washing with antiseptic solutions before the swab is taken may hinder the growth of the offending microbe on the bacteriologist's media. Where time and circumstances make it practicable, pathologists will prefer personally to swab the throat.

The usual full routine examination of a swab calls for a gram-stained smear (rather intensely counterstained with dilute carbol-fuchsin), culture on blood-agar for streptococci, etc. and culture on Loeffler slope and tellurite plate for the diphtheria family.

Vincent's Disease

Vincent's bacilli if present are readily seen as gram-negative fusiform rods often barred, with numerous gram-negative spirochaetes. Although this diagnosis will enable the clinician to prescribe at once whatever treatment he favours, the rest of the routine examination should be completed, for Vincent's organisms may be abundantly present as secondary invaders of lesions due to glandular fever (infectious mononucleosis), agranulocytosis and leukemia. They may be rather scantily present in other throat infections and it is helpful to the clinician to indicate how numerous these organisms are in the smear. Thus the diagnosis of Vincent's angina as the main or sole disease present must rest with the clinician. If, despite a report of numerous Vincent's organisms, the doctor is dubious about the diagnosis, a blood-count may be of considerable help.

Streptococcal Infection

The Blood plate may give a profuse growth of many organisms with the haemolytic streptococcus as the principal pathogen. It is useful to indicate roughly the proportion of haemolytic streptococci relative to other bacteria, *e.g.* 20, 50, 80 per cent. Since this organism may be overgrown and not readily seen on the blood agar plate, it may be advisable, particularly in the search for carriers to include a blood plate designed to favour streptococci and restrict staphylococcus and other organisms. Crystal violet, 1-500,000

incorporated in the agar (or a few drops of 1 : 3,000 dilution spread over the plate which is then dried just before inoculation), will often reveal haemolytic streptococci with clear zones of haemolysis which might have been missed on the plain blood agar. Some streptococci give better haemolysis on the blood plate if incubated anaerobically, and some strains may fail to produce any zone of haemolysis or at most some green pigment. On the other hand, the bacteriologist must keep in mind haemolytic variants of the influenza bacillus which may superficially resemble colonies of haemolytic streptococci. Therefore until experience has been gained, it is wise to confirm that the colony producing the haemolysis is really a beta haemolytic streptococcus by smear, and, if necessary, other tests.

Some workers always test the power of the streptococci to produce soluble haemolysis by the "tube test." A single colony is transferred into 20 per cent serum broth and incubated overnight. One cc added to one cc of 5 per cent. washed horse-red-cells is kept at 37° C for an hour, positive cultures lyse the red cells. In the ordinary routine of a clinical laboratory, plate-positive but tube-negative cultures from "sore throats" are rare.

When the pathologist is trying to trace infection from one person to another, he will test for the Lancefield grouping of the streptococci isolated, and for the Griffith type (see pp. 32, 36).

Diphtheria

In the search for diphtheria bacilli the aim should be speed and accuracy of diagnosis, and secondly the typing of the corynebacterium present. Examination of direct smears for the diagnosis of diphtheria is not to be recommended, since even in experienced hands it does not give more than 30-40 per cent. of positive results. For speed Loeffler tubes or plates have long been used and still are in many laboratories. The maker of the Loeffler medium will bear in mind such a paper as that by Goldsworthy and Wilson (1942). They used, in parallel tests, Loeffler media made in several different laboratories. The "worst" Loeffler detected only 30 per cent. of those revealed by the "best," the main cause of spoiling of the medium being overheating. These authors write: "Mix 4 parts of filtered sterile horse-serum with 1 part of sterile 1 per cent glucose broth, tube aseptically in sterile test-tubes and place in the inspissator at 85° C. for 2 hours. The addition of 5 per cent. glycerol improves granule production in both *C. diphtheriae* and other organisms." The great advantage of the Loeffler

medium is that the diphtheria bacilli, if present in numbers, grow well overnight and show typical morphology and staining under the microscope. A further examination on the second day will increase the number of positive results. For staining, many modifications (Neisser's etc.) founded on alkaline methylene blue are in use. Albert's toluidine-malachite-green stain is widely used and gives excellent results (see Appendix). Much caution is advisable in using Loeffler media sown with material from nose, ears, vagina or wounds. These may yield bacilli morphologically indistinguishable from those of true diphtheria which are non-toxigenic and are regarded as non-pathogenic. Tellurite plates and fermentation reactions differentiate them.

A great improvement resulted from the introduction of the potassium tellurite media. Those bacilli which convert tellurite to the black telluric acid give immediate indication of their presence. There is no general agreement yet as to the simplest and most useful tellurite plate. The ideal medium should be easy to make, of constant composition and appearance, allowing rapid growth of all types of diphtheria bacilli which would show typical staining reactions on the microscopic slide, would suppress all but diphtheria bacilli and would give characteristic colonies enabling the pathologist at once to identify the *gravis*, *intermedius*, or *mitis* group. Large numbers of modifications have been described; some workers favour the opaque heated blood agar originally used by McLeod, others prefer unheated or lysed blood added to the agar.

What is the "best" medium is at present largely a matter of personal opinion. Media that have proved useful, are easily prepared and are widely used in Britain are Hoyle's laked blood medium and Neill's heated blood medium (see Mackie and McCartney, 1945). Heated blood-agar while very good for colonial differentiation develops the substance "haem" which may be inhibitory to certain *mitis* and *gravis* strains. An unheated blood medium (originally used by Professor A. W. Downie) which is easy to prepare and gives colonies which with experience can be recognised at 18-24 hours is described in the appendix.

Colonies in these plates are examined after 18 and 24 hours at 37° C. Plates should be given a further 24 hours' incubation and examined at 48 hours. The colonies have a different characteristic appearance at each of these times. Smears stained with methylene blue show typical morphology both at 24 and 48 hours. A description of the colonial appearance of *C. diphtheriae* to be seen on the

various culture media; the worker without much experience should train his eyes by obtaining colonies of the three standard types of diphtheria bacilli and plating them until he is satisfied. He can then keep cultures of the three types available and culture them on his own plates in parallel with the bacilli isolated from patients and so confirm the identification of type.¹

The colonies on the Downie plates appear as follows :

1. *gravis* 24 hrs. Approx 1-2 mm. circular, entire edge, matt surface, grey in colour. Occasional strains may show a very faint haemolysis round each colony.
48 hrs. 2-4 mm. Very dark grey almost black Central papilla, radial striations, lobate edge, matt, typical "daisy head" not always present.
2. *mitis* 24 hrs. Small, 1-2 mm. Pale grey, circular, entire edge. A well marked zone of haemolysis.
48 hrs. 2-4 mm Circular, entire, black shiny centre with greyish periphery.
3. *intermedius* 24 hrs. $\frac{1}{2}$ -1 mm. Flat, greyish, translucent with shiny surface.
48 hrs 1 mm. Flat with black central plateau and splayed grey periphery.

Most pathologists, even after much experience with the tellurite medium they have chosen, confirm their cultures. The routine of a given laboratory might run as follows :

The swab is received in the morning. If a gram-stained smear reveals numerous gram-negative Vincent's bacilli and spirochaetes, that report is immediately telephoned to the clinician.

The swab is incubated on Loeffler, blood-plate and tellurite plate.

Next morning (or in urgent cases, late the same afternoon) the Loeffler culture is examined, the whole growth being emulsified and Albert's stain used. If typical diphtheria bacilli are seen, a provisional verdict is reported at once (unless the swab is from nose or ear).

On the tellurite plate a typical colony is examined with the plate microscope and is transferred to 5 per cent. serum agar. After about 7 hours, i.e. about 5 P.M., the serum agar culture is sown in glucose, saccharose, and starch serum peptone water (see Appendix). A blood plate may also be sown to control purity of the colony picked from tellurite, in case the fermentation results are anomalous.

¹ *Stock Cultures.* Cultures of the three types of diphtheria bacilli can be obtained from The National Collection, Lister Institute, Elstree, Herts.

medium is that the diphtheria bacilli, if present, will overnight and show typical morphology at the microscope. A further examination on the medium will increase the number of positive results. For special examinations (Neisser's etc.) founded on alkaline medium use. Albert's toluidine-malachite-green stain gives excellent results (see Appendix). Much can be done in using Loeffler media sown with material from exudates or wounds. These may yield bacilli morphologically distinguishable from those of true diphtheria which are and are regarded as non-pathogenic. Tellurite and tellurite reactions differentiate them.

A great improvement resulted from the introduction of sium tellurite media. Those bacilli which give a black telluric acid give immediate indication of pathogenicity. There is no general agreement yet as to the ideal tellurite plate. The ideal medium should be of constant composition and appearance, allow for the growth of types of diphtheria bacilli which would give different reactions on the microscopic slide, would suppress other bacilli and would give characteristic colour reactions at once to identify the *gravis*, *mitis*, and *conglutina*. Large numbers of modifications have been suggested, but favour the opaque heated blood agar or others prefer unheated or lysed blood added.

What is the "best" medium is at present a personal opinion. Media that have proved themselves and are widely used in Britain are Loeffler's medium and Neill's heated blood medium (Neill, 1945). Heated blood-agar while it is used for differentiation develops the substance which is inhibitory to certain *mitis* and *gravis* strains. Loeffler's medium (originally used by Professor Loeffler) is easy to prepare and gives colonies which are easily recognised at 18-24 hours is described in the Appendix.

Colonies in these plates are examined at 18-24 hours at 37° C. Plates should be given a further examination at 48 hours. The colonies have a characteristic appearance at each of these times. The colonies on blue show a characteristic morphology by the 48 hours.

It is difficult to give a short summary of the different types of *C. diphtheriae*.

were released within the ensuing month. The important point is that though the Loeffler failed to reveal about 50 per cent. of those giving a moderate number of colonies on tellurite, it also failed with approximately 25 per cent. of those giving heavy growth on tellurite. It is important that a negative result on a tellurite plate should be followed as quickly as possible by another test, thus reducing the exposure to possible further cross-infection from other patients.

In the search for carriers when a case of diphtheria has occurred in a ward or institution, culture of both nose and throat swabs on tellurite medium is the surest and quickest method of detecting a possible source of infection.

With tellurite media also the *type* of diphtheria bacillus can be identified. Since *gravis* and *intermedius* strains are almost always virulent, *mitis* is the only one needing a virulence test in routine work. The two former types usually cause more severe infection with perhaps a 10-15 per cent. of incidence of paralysis; they therefore call for even greater nursing care. Typing of the diphtheria bacilli has also considerable epidemiological value since it helps in tracing sources of infection and in providing an explanation of variations in morbidity and mortality in different areas and in the same area in different years.

It cannot too often be reiterated that "When a sore throat justifies a swab, it also justifies prophylactic diphtheria antitoxin without awaiting a report." Diphtheria is a clinical entity, not a bacteriological finding. The old fear of reactions after prophylactic antitoxin has virtually no foundation if modern enzyme-treated ("refined") antitoxin be used.

Appendix

Albert's Stain: Laybourn's modification, in which malachite green is substituted for methyl-green, is given here instead of the original method.

Solution 1:

Toluiden blue	.	.	0.15 g
Malachite green	.	.	0.2 g
Glacial acetic acid	.	.	1 cc.
Alcohol (95 per cent)	.	.	2 cc
Distilled water	.	.	100 cc

Dissolve the dyes in the alcohol and add to the water and acetic acid. Allow to stand for one day and then filter.

Solution 2 :

Iodine	2 g.
Potassium iodide	3 g.
Distilled water	300 cc.

Note :—The iodine solution used in Jensen's modification of Gram's method works equally well.

Technique :—

- Make film preparation and fix by heat.
- Apply solution 1 for three to five minutes.
- Wash in water and blot dry.
- Apply solution 2 for one minute.
- Wash and dry

By this method the granules stain bluish black, the protoplasm green and other organisms mostly light green.

This method can be recommended for routine use.

Blood-tellurite Medium :

- 400 g fat-free minced bullock's heart.
- 4 g anhydrous Na_2CO_3 .
- 2.5 g. NaCl .
- Tap water 1000 cc.

Infuse overnight in ice chest.

Add 8 cc concentrated HCl , and bring to boil over flame.

Squeeze and filter (Green's filter paper 904½).

Add 25 g. shred agar Autoclave at 15 lb. for 15 minutes.

Add 10 g. Evans peptone or Difco Proteose peptone.

Adjust pH to 8.2 (cold), bottle and autoclave at 10 lb. for 10 minutes

After the final autoclaving the pH falls to about 7.6.

For use the agar is melted, decanted from the phosphate deposit, the pH checked and adjusted if necessary, and poured as two-layered plates. A thin layer of nutrient agar is first poured and allowed to set. To the top later are added 5 per cent. horse blood (oxalated or defibrinated) and 0.04 per cent. potassium tellurite (from 2 per cent. recently prepared but unsterilised solution).

Serum peptone water base for sugars: Dissolve 7 grams peptone (Difco proteose or Evans) and 1.4 grams Na_2HPO_4 in 1,400 cc. distilled water.

Steam 15 minutes and filter through paper. Adjust pH to 7.4.

Add 250 cc. horse serum and steam for 20 minutes.

Add 11 cc Andrade's indicator. pH should now be 7.6-7.8.

Tube in 3 cc. quantities in 6' × ½" tubes and autoclave at 10 lb for 10 minutes.

It is an advantage to have a perfectly clear medium. Different batches of serum differ in their coagulability by heat and occasionally

the medium is cloudy where serum in the amount given above is used. It is a good plan to add varying amounts of each batch of serum to the basal medium in test tubes (to give concentrations of 10-20 per cent.) and autoclave, choosing the highest concentration that shows no marked cloudiness afterwards

Starch (B.D.H. soluble or potato). Make a 2½ per cent solution in distilled water and sterilize in the autoclave at 10 lb. for 10 minutes

Add 0.6 cc. of the sterile 2½ per cent solution aseptically to 3 cc of serum water.

Other Sugars: Make a 10 per cent. solution, sterilize by filtration or autoclaving for 10 minutes at 10 lb and add 0.3 cc. to 3 cc. of serum water.

Before making up large batches preliminary tests may with advantage be carried out as follows —

- (a) On the serum water without added carbohydrate to ensure that no fermentable substance is present
- (b) On the soluble starch medium with known *mitis*, *intermedius*, and *gravis* strains. This need only be done with each new batch of starch, to ensure that the process of acid hydrolysis used in manufacture has not been carried too far.
- (c) On each new batch of sucrose using *gravis*, *mitis* and *intermedius* strains and a known sucrose fermenting diphtheroid, to ensure that hydrolysis has not occurred, and that no extraneous fermentable substance is present.

R. A. O'BRIEN.

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CHAPTER IV

SEROLOGICAL CLASSIFICATION OF STREPTOCOCCI

Introduction. The first successful classification of the streptococci was founded on the basis of their haemolytic activities, but while in the vast majority of human infections a streptococcus which produces a zone of β -haemolysis on a blood-agar plate may be safely regarded as a *Streptococcus pyogenes*, it must be borne in mind that strains are occasionally encountered which, while possessing a soluble haemolysin, display only very feeble haemolysis on the surface of a solid medium. Such strains, which produce a zone of greenish discoloration on blood-agar, have been shown by Todd (1928) and Fry (1933) to be variants of β -haemolytic streptococci, and a zone of frank haemolysis may be obtained by growing the organisms anaerobically or in the depths of a blood-agar plate. In routine work they are unlikely to be mistaken for *Str. viridans* if in sowing the plate the platinum loop is dug under the surface of a part of the medium, where the resulting deep colonies will indicate the true nature of the organism.

On the other hand, some streptococci which are β -haemolytic on blood-agar appear unable to produce a soluble haemolysin, and such strains are commonly regarded as of doubtful pathogenicity, at least so far as man is concerned. It is useful, therefore, in the investigation of outbreaks of streptococcal infection, to test streptococci isolated from supposed carriers for the presence of a soluble haemolysin before having these organisms subjected to the serological grouping and typing presently to be described. A haemolytic colony is picked from a plate and inoculated into 20 per cent. horse-serum broth, which is incubated for not more than 18 hours. (After 18 hours the haemolysin formed undergoes rapid deterioration.) 0.5 cc of the broth-culture is added to 0.5 cc. of a 5 per cent. suspension in normal saline of horse red cells and the mixture placed in a water-bath or incubator at 37° C. for one hour, by which time haemolysis should be complete. If a rough quantitative estimation of haemolysin-production is required, varying amounts of the culture (e.g. 0.1 to 1 cc.) may be added to a series of tubes containing 0.5 cc of the red cell suspension.

Lancefield Groups

The division of streptococci by Lancefield (1933) into groups by means of precipitation reactions with specific antisera is a valuable

means of assessing the significance of strains isolated from human infections. These precipitation reactions depend upon group-specific carbohydrates, which appear to be haptens, as they are antigenic only when still in combination with the cellular protein.¹ The group-specific carbohydrate appears to form an integral part of the bacterial cell, since methods involving cellular disruption are required to obtain it in any quantity. Lancefield's method of extraction by acid-hydrolysis leaves a large residue of undissolved bacteria, and requires 10-50 cc. of broth culture. The method described by Fuller (1938), in which the bacteria are completely dissolved by formamide, provides an equally satisfactory extract using only 5 cc. of culture.

Technique

Lancefield's Method—50 cc of an overnight culture of the organism in 0.05 per cent glucose-broth is centrifuged, the deposit suspended in 1 cc. of N/20 HCl, two drops of 0.2 per cent. Congo red solution added, and the mixture further acidified, if necessary, with one or two drops of N/1 HCl until a slate-blue colour is obtained. It is then heated in a water-bath at 100° C for 10 minutes, cooled and centrifuged. The deposit is discarded, and the supernatant fluid, to which a drop of phenol red solution has been added, is neutralized with N/1 NaOH and recentrifuged. The resulting clear supernatant fluid constitutes the antigenic extract.

A more rapid method is to suspend a third or more of the colonies on a blood-agar plate in 0.15 cc. N/10 HCl; boil and centrifuge as above, and use the supernatant fluid for the precipitin test.

Fuller's Method—0.1 cc. of formamide is added to the centrifuged deposit from 5 cc. of an 18-hour glucose-broth culture. The tube is thoroughly shaken and placed in an oil-bath at 150° C. for 15 minutes (A small enamel mug containing olive oil or liquid paraffin will serve for an oil bath, and a careful watch must be kept to ensure that the temperature does not vary more than 5° C. on either side of 150° C. The temperature of extraction is not critical, but above 150° C. there is a tendency to charring, and below it the bacteria dissolve very slowly.) Cool and add 0.25 cc. of acid-alcohol—95 parts of absolute alcohol with 5 parts of 2N hydrochloric acid (1 part of concentrated HCl to 4 parts of water.). Mix, centrifuge, remove the supernatant fluid to another tube and add

¹ Satisfactory group-specific antisera (A, B, C, D, G) are now obtainable from Burroughs, Wellcome & Co., and the test can be easily carried out even in small laboratories.

0.5 cc. of acetone. Shake the tube, centrifuge and discard the supernatant fluid, draining off as much acetone as possible. Add 0.7 cc. of normal saline to the deposit, shake thoroughly, add a drop of phenol red solution, and make slightly alkaline with a drop or two of 0.5 per cent. sodium carbonate. This method can be strongly recommended as providing a reliable extract quickly prepared from a small amount of culture. If the growth of haemolytic streptococci on the original plate is profuse, the grouping result can be reported on the same day by scraping haemolytic streptococci off the plate into 0.1 cc. of formamide and preparing the extract as above.

In the performance of the precipitation test, capillary tubes about 7 cm. long and of 2 mm. bore may be used. A column of Group A serum 1.5 cm. long is drawn into the capillary and allowed to run more than halfway up the tube, the lower end of which is then sealed by heat. An approximately equal volume of antigenic extract is layered on to the serum by means of a fine capillary pipette, and the tube placed upright in a plasticine-filled groove made in a block of wood. (Alternatively, the test may be made in a Pasteur pipette cut down so that the capillary part measures 6-8 cm. and the expanded part about 1.5 cm. The serum is allowed to run up almost to the base of the expanded part and the antigen layered on with an ordinary Pasteur pipette. This method is easier, and the cut-down pipette can be cleaned and used several times.) The precipitin reaction can be observed as an opaque ring at the interface of serum and extract within a few minutes, and certainly within 30 minutes at 37° C. If left overnight cross-reactions may occur or the precipitate dissolve. The tests should be set up using antisera for Groups A, B, C and G.

Interpretation

Of the streptococci classified by Lancefield, only Groups A, C and G characteristically possess a true haemolysin. (Non-haemolytic strains belonging to Group A are occasionally met with feebly haemolytic or "green-producing" strains have been mentioned above.) Group B streptococci may be almost non-haemolytic when grown aerobically on blood-agar plates, but are often frankly haemolytic under anaerobic conditions; only a proportion of Group D strains possess a true haemolysin. Group D strains, which are haemolytic enterococci, show β -haemolysis on blood agar, but do not produce a soluble haemolysin. Of the strains infecting human subject the great majority belong to Group A. In a series of 1,283 strains of haemolytic streptococci isolated from the throats

in acute upper respiratory infection, where 20 per cent. or more of the plate-growth consisted of haemolytic streptococci, Allison (1942a) found that 89.48 per cent. belonged to Group A, 7.48 per cent. to Group C, 2.96 per cent. to Group G, and 0.08 per cent. to Group B. Schwentker, Janney and Gordon (1943), in a study of 3,080 strains isolated from the throat of normal carriers in Rumanian villages at a time when there was no scarlet fever, found that 49.8 per cent. were Group A, 33.5 per cent. Group C, and 12.8 per cent. Group G. (The remaining 3.9 per cent. were reported as not belonging to Groups A, C or G)

Comparison of these two sets of figures indicates that Group A strains are by far the commonest cause of upper respiratory tract infection, and that Group C and Group G strains are responsible for only a small proportion of such cases, though they are not infrequently met with in cultures from normal throats. Group C strains have also been reported as causal agents in erysipelas and cellulitis. Group B streptococci are very rarely found in the throat, though, owing to their poorly haemolytic properties under aerobic conditions, it is possible that some of these strains are missed in the examination of blood-agar plates.

In puerperal infections associated with the haemolytic streptococcus, Group A strains provide the great majority of cases, but the incidence of strains other than Group A is by no means negligible. Ramsay and Gillespie (1941) in a series of 305 cases of haemolytic streptococcal infection in a puerperal sepsis unit found Group B strains in 5 per cent., Group C strains in 4 per cent., Group D strains in 2 per cent. and Group G strains in 4 per cent., and though these infections were usually mild, they included three fatal cases of acute endocarditis, two caused by Group B streptococci and one by a Group G streptococcus. Streptococci of groups other than A were isolated from a much higher proportion of post-abortum than of post-partum streptococcal infections (43.1 per cent. compared with 9.3 per cent.). In half the cases where Group D streptococci were isolated it was uncertain whether these were the cause of the sepsis, and organisms of this group are regarded as of low or doubtful pathogenicity.

Of other groups so far distinguished, E is found in normal milk, F, H and K in human throats as commensals, and L and M in canine infections.

To sum up, while haemolytic streptococci belonging to Groups B, C and G occasionally cause infection in man, those of Group A are by far the most frequent human pathogens and of the greatest epidemiological importance. Carriers of Group A streptococci,

always a potential source of infection, are particularly likely to prove dangerous if employed as midwives or surgical dressers, and should therefore be prohibited from attending on parturient or surgical cases while they are harbouring these organisms. Carriers of other than Group A haemolytic streptococci need not be subjected to any restriction other than the wearing of an efficient mask over the nose and throat, as such strains are usually of low infectivity.

Griffith Types

The chief value of Lancefield grouping lies in indicating sources of potential danger, but since it does not distinguish between strains in a particular group it is of little help in tracing the origin of an actual outbreak of streptococcal infection where Group A streptococci may be isolated from several members of the nursing or medical staff. Griffith (1934) divided Group A streptococci into a number of serologically distinct types, and a very large proportion of strains from human infections can now be identified with certainty.

By the use of agglutinin-absorption methods Griffith prepared specific agglutinating antisera against 30 serological types of streptococci. (Of these all but four belong to Group A; of the rest, Types 7, 20 and 21 belong to Group C, while Type 16 belongs to Group G.) The test is carried out by direct slide-agglutination, using first pooled sera (each pool representing five types), and then using the individual sera comprising the pool which has given an agglutination reaction. The two chief difficulties involved in the test are (a) the obtaining of a stable suspension of organisms, and (b) the occurrence of cross-reactions.

(a) Frequently streptococci grown in Hartley broth with or without the addition of serum give "granular" suspensions in which the organisms occur in clumps and which are therefore unsuitable for agglutination tests. Various methods have been suggested to overcome this difficulty; e.g. growing the organisms in 0.2 per cent. glucose-broth at 22° C., subculturing the supernatant fluid after the granular suspension has settled and repeating the process until an even suspension is obtained, and treating the granular deposit for varying periods (one hour to 18 hours) with trypsin. Perhaps the best way to obtain an even and stable suspension on primary fluid-culture is to grow the organisms in a quick-growing medium, e.g. veal-broth, which gives successful results with about 80 per cent. of epidemic strains, though strains isolated from sporadic infections or from carriers present greater difficulty.

(b) It is sometimes found that a streptococcal suspension will agglutinate with more than one of the Griffith type-sera. This may not constitute a problem in well-defined outbreaks where the same cross-reactions are obtained with all the strains isolated, but in sporadic cases there is often difficulty in assigning such an organism to a particular type. Lancefield has explained these cross-reactions by showing that at least two type-specific antigenic components, designated M and T, are concerned with the agglutination reaction. M, a type-specific protein, is the antigen primarily associated with virulence; the antibody to which it gives rise provides specific protection in experimental animals against the homologous organism and also plays some part in the agglutination reaction. The T-substance, the nature of which is at present undetermined, is the most important type-specific agglutinin concerned in the agglutination reaction, but antibodies to it play no part in the development of protective immunity. In most strains there is a definite correspondence between the M and T antigens, but, while members of each type elaborate a distinct type-specific M-substance, it sometimes happens that an organism may possess the T antigen of another type (or types) in addition to its own, and these extra T-substances cause cross-reactions with heterologous sera. Elhott (1943) has suggested that strains possessing M and T antigens may be subject to a form of diphasic variation, either antigen being dominant when the other is recessive; in the types he studied the M-phase appeared to be favoured by growth at 37° C., and the T-phase by growth at 22° C. Lancefield has shown that the M-substance can withstand boiling for 15 minutes, but is quickly digested by trypsin, whereas the T-antigen resists treatment by trypsin but is destroyed by boiling. When, therefore, a strain gives cross-reactions with several sera, its true type may be discovered by heat-treatment, when only the type-specific M-antigen should remain.

The presence of anomalous cross-reactions often causes delay in assigning an organism to its type, and to obviate this, Swift, Wilson and Lancefield (1943) have introduced a typing method in which bacterial extracts containing the M-antigen react in the precipitin test with type-specific anti-M sera.¹ In this test only a single

¹ Elhott (1945) has recently shown that Group A streptococci sometimes produce in broth-culture an extracellular proteolytic enzyme which completely destroys the M-antigen in cultures grown at 37° C., though some M-substance remains in cultures grown at 22° C. Production of the enzyme can be permanently suppressed by serial passage through mice.

antigen-antibody system is concerned, there is practically no crossing among types, and there is no need to obtain an even bacterial suspension as in the agglutination test.

Of the two methods of typing, that of Griffith is predominantly used in Britain, and in the hands of experienced workers can give reliable results in the great majority of epidemiological investigations, such as the study of institutional outbreaks of scarlet fever, puerperal sepsis or other streptococcal infection. By the use of Griffith typing it has been repeatedly shown that infection with one serological type of streptococcus gives no protection against infection with another type; e.g. the so-called "relapse" in scarlet fever is really a fresh infection (in a patient who has failed to develop immunity to the erythrogenic toxin) by another type of *Str. pyogenes*, the source of which can almost always be traced to another patient or member of the ward staff; the later complications of scarlet fever are also very often caused by strains which differ serologically from the primary infecting type. A comprehensive survey of such investigations is given by Allison (1942b).

Griffith typing sera are not at present manufactured for general distribution, but the Streptococcus Reference Laboratory of the Central Public Health Laboratory, Colindale Avenue, London, N.W.9 is available for consultation in matters concerning the investigation of outbreaks of streptococcal infection.

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CHAPTER V

LABORATORY DIAGNOSIS OF PRIMARY ATYPICAL PNEUMONIA

It has become increasingly evident, particularly during the years since sulphonamide drugs have been in general use, that certain outbreaks and sporadic cases of pneumonia fail to conform either clinically or bacteriologically to the accepted picture of pneumococcal pneumonia, and do not fall easily into the category of "broncho-pneumonia." These primary "atypical" pneumonias are usually mild, but they are certainly infectious, and their exact aetiological diagnosis presents the laboratory with a difficult problem. The earliest reports in England came from Gill (1938), and Ramsay and Scadding (1939), but few detailed studies have been made except in the United States of America (Reimann, 1943, Dingle *et al.*, 1944). No bacterial species has been incriminated, but in some series by animal inoculation of human material and by complement fixation techniques using acute and convalescent sera, evidence of infection with viruses of the psittacosis group has been obtained in 3-15 per cent of cases. Failure to infect a wide variety of experimental animals has been the rule, but Eaton *et al.* (1944) have produced lung lesions in cotton rats by intranasal instillation of sputum and lung tissue, either directly or after passage in the chick embryo. Unequivocal evidence of virus aetiology has been provided by the American Commission on Acute Respiratory Diseases (Dingle *et al.*, 1945) which reports the successful transfer of infection by filtered sputum and nasal washings to three out of twelve volunteers; these developed clinically typical attacks accompanied by the appearance of cold agglutinins in the serum.

The first thorough study of cold agglutinins in the serum of patients suffering from "an unusual form of broncho-pneumonia" was made by Clough and Richter (1918). More recently accounts have appeared in England (Turner *et al.*, 1943); America (Petersen *et al.*, 1943) and India (Shane and Passmore, 1943). Turner's observations were made among a fairly homogeneous group of men in the prime of life, in whom the pneumonia presented a clearly recognizable clinical entity. Of 83 unselected cases more than half had an agglutinin titre of 1:32, while the sera of 23 showed titres of 1:128 and higher. Of the control group of 132 patients suffering from a variety of other respiratory diseases, only 5 had sera with a titre exceeding 1:16, which he considered to be the maximum

normal level using his technique. The titre of cold agglutinins rose to a peak between the 10th and the 20th day of illness, but the height reached bore no apparent relationship to the severity of the disease, to sulphonamide therapy or to the presence of pathogenic bacteria in the sputum. During convalescence there was a gradual decline.

Technique. Several techniques are in use, based upon the same principle but using different proportions of reagents and with different end-points. The precautions to be observed are the same for each. Blood is allowed to clot at 37°C ., and the serum is removed at room temperature or, better, at 37°C . The serum can now be stored in a refrigerator, but it should be tested within a week, since marked falls in titre have been described after the serum has stood at 4°C . The test cells should be fresh, not bacterially contaminated, and should be either of group "O" (IV) or from the patient himself, in order to avoid iso-agglutination. They should be washed twice at room temperature with isotonic NaCl solution before use.

Serial twofold dilutions of serum in isotonic salt solution are made in Wassermann tubes. To each tube is added an equal volume of a 1% suspension of the test cells in isotonic saline. The tubes are shaken and left overnight at 0 to 4°C . Readings are made while the tubes are still cold, by gently tapping the tube to re-suspend the cells. The degree of agglutination is noted as 4+ to 0; 4+ being recorded when all the cells remain agglutinated in a solid clump. This gives the macroscopic end-point. The microscopic end-point can be observed by rapid examination on cold slides, and is usually one or two tubes higher. Only the macroscopic end-point has been considered in studies of atypical pneumonia. An initial serum titre of 1:32 or over during a suitable stage of the disease is taken as a positive result. The phenomenon is true cold agglutination and disappears when the tubes are transferred to a bath at 37°C .

Interpretation. A comprehensive review of cold haemagglutination has been made by Stats and Wassermann (1944). High titres are not common, and there is no doubt that the observations in atypical pneumonia are significant, but since cold agglutinins have been observed in a variety of other conditions, these must always be borne in mind; the most important are cirrhosis or syphilis of the liver, leukaemia, trypanosomiasis, haemolytic anaemia and Addisonian anaemia, staphylococcal septicaemia, and in association with Raynaud's syndrome and with sulphonamide therapy; trypanosomiasis is the only one of these diseases in which marked cold agglutination is the rule (Yorke, 1911). Raynaud's syndrome,

acrocyanosis, and even haemolytic anaemia have been observed to occur during the course of atypical pneumonia in association with a high titre of cold agglutinins, and in these cases the pneumonia was probably the primary disease.

The reason for the development of cold agglutinins is not known. They may be a response to infection with a specific virus sharing an antigenic component with erythrocytes, or be analogous to the "auto-antibodies" formed in trypanosomiasis. It has been suggested that the auto-antibody results from "auto-immunisation" by substances released from the altered tissues possibly loosely combined with protein from the trypanosomes.

Two observations interesting in this connection have been made in other outbreaks of atypical pneumonia. The first is the development in certain sera during the course of the disease of the property of fixing complement with crude suspensions of various tissues from small laboratory animals. The second is the appearance in certain persons of agglutinins against a particular non-haemolytic streptococcus MG, first encountered at autopsy in the lungs of two fatal cases, but the connection of which with the disease has not yet been proved (Mirick *et al.*, 1944). There is at present no evidence linking any of the three phenomena together.

Other Laboratory Findings in Atypical Pneumonia. In most of the series of primary atypical pneumonia cold agglutination tests have not been recorded, but there are nevertheless numerous cases thus diagnosed in which the sera has shown no abnormal rise of agglutinins. It is pertinent therefore to ask upon what the diagnosis is to be based, and whether a number of aetiologically different conditions may not fall under this heading. Clinically primary atypical pneumonia is an infectious disease in which there are radiological pulmonary changes corresponding neither to the usual picture of lobar nor of broncho-pneumonia. The disease is usually mild and only rarely fatal, and has hitherto been chiefly observed in army training camps where men even with mild illness nevertheless receive a thorough examination. Its course is unaffected by sulphonamide therapy in doses adequate to treat pneumococcal pneumonia, and the temperature falls to normal in 1-4 weeks. It is often preceded by headache and sore throat. Cough is unproductive, and the accepted pathogenic bacteria are usually absent from the sputum. Leucocyte counts of the blood show no leucocytosis but are usually normal or low, possibly with some increase in lymphocytes. Stained smears of sputum may show a predominance of mononuclear cells in the early stages.

Such a clinical picture clearly could, and does appear in virus

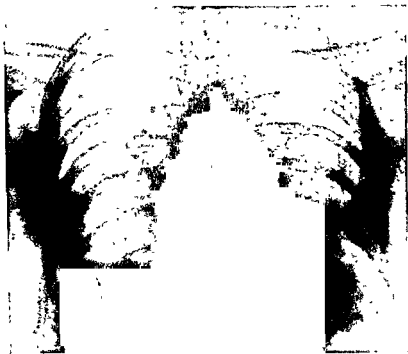
infections of the psittacosis and other recognised groups as well as primary atypical pneumonia of unknown aetiology; it may also occur in atypical cases of pneumococcal pneumonia, which are not uncommon particularly when the disease picture is modified by administration of drugs of the sulphonamide series. Small sterile pleural effusions are present in many such treated pneumonia cases and are quite commonly accompanied by some pyrexia. Even before the administration of sulphonamides there may be no leucocytosis or even a leukopaenia, particularly in Type III pneumococcus infections which may also give a confusing radiological picture, but which are usually severe. Much depends therefore upon the sputum examination and its interpretation. In pneumococcal pneumonia pneumococci are often scanty before the crisis and may remain so throughout under continuous sulphonamide therapy. (This is not due to the inhibitory effect of sulphonamides in the sputum, since pneumococci can readily be recovered from sputa with high sulphonamide content provided that they were present initially.) More than one sputum sample may have to be examined and mouse inoculation performed in order to demonstrate pneumococci; if they are present only in small numbers their significance cannot be assessed without serological typing, for the carrier rate in normal individuals is high. Pneumococci of Types I and II are uncommon in healthy persons, and since these types, together with Types III, V, VII, VIII and XIV are the commonest causes of primary pneumococcal pneumonia their presence is usually significant. The only absolute proof is the demonstration of a marked increase in antibodies against the suspected organism during the course of the disease, but such proof is not easily demonstrated. In sporadic cases of atypical pneumonia the exclusion of a pneumococcal aetiology can seldom be done by the laboratory or the clinician alone, but in outbreaks in fairly closed communities the presence of primary atypical pneumonia is much more readily recognised, and here the observation of cold agglutinins in the serum is of considerable help in diagnosis.

X-ray Findings. The recognition of primary atypical pneumonia depends largely upon radiological findings. These can be correlated with the picture revealed at autopsy, which is one of acute bronchiolitis, usually focal in distribution (Golden, 1944). Damaged bronchioles are commonly filled with sterile pus and desquamated epithelium, their walls are infiltrated with mononuclear cells, and this infiltration extends into the surrounding lung tissue. The alveoli beyond may either be collapsed or contain air. In severe cases secondary bacterial infection may produce a picture





A Primary Optical Pneumonia
8th day of disease (for description see p. 43)



B Primary Atypical Pneumonia
18th day of disease (subsequently died) (for description see p. 43)

very like that of broncho-pneumonia. As would be expected from such a pathological picture the radiological findings are rather diverse and not entirely specific.

The most common radiographic appearance is of an affected area showing marked reticulation. This reticulation extends from the hilum, where it is usually maximal, to the periphery (Plate I, A, B). It is most frequently seen in the lower lobes, but may involve any part of the lung. In some cases the reticulated area shows miliary nodules radiologically almost indistinguishable from those seen in silicosis. A less common appearance is that described by Drew *et al.* (1943) of a diffuse loss of translucency at the periphery giving a ground-glass appearance within which can be seen the same finely nodular opacities. This loss of translucency is less marked than that seen in lobar pneumonia and the part involved is usually separated from enlarged hilar glands by a somewhat clearer area of lung tissue (Plate I, B). When such radiological appearances involve only a small area of the lung field the picture is easily confused with that of early tuberculosis and it is only by its transient nature that the differential diagnosis can be made. Super-added infection will convert either of these pictures into one indistinguishable from that of broncho-pneumonia.

The radiological abnormalities are usually much more extensive than physical signs in the chest would indicate.

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CHAPTER VI

LABORATORY DIAGNOSIS OF PERTUSSIS

PERTUSSIS is a difficult disease to diagnose clinically in the early stages of the infection ; yet early diagnosis is most desirable for the proper care and nursing of the affected child and for the prevention of spread of the infection. The only reliable diagnostic test at this early stage of the infection is the isolation of the infecting organism *Haemophilus pertussis*, but a leukocytosis with relative lymphocytosis in the presence of a suspicious spasmodic cough will give strong presumptive evidence of the infection.

BACTERIOLOGICAL DIAGNOSIS

Acceptance of the isolation of *H. pertussis* from affected patients as diagnostic of whooping-cough assumes that this organism is the sole aetiological agent, and the following criteria in favour of such an assumption may be quoted

(1) *H. pertussis*, a small gram-negative cocco-bacillus, has been isolated from over 90 per cent. of patients in the early infectious stage of the disease and but rarely from convalescents or unaffected persons. (2) With pure cultures of the organism the infection has been reproduced in human volunteers and in apes, whereas this was not achieved with culture filtrates. (3) Specific antibodies to *H. pertussis* develop in the blood of naturally or experimentally infected individuals, humans or other animals in which such antibodies are demonstrable after vaccination are resistant to attack. (4) Attempts to establish a filtrable virus as the cause of pertussis have failed.

For success in the isolation of *H. pertussis*, a reliable culture medium (see Appendix) and some experience in the recommended technique are necessary. It must also be remembered that in hospital practice, perhaps 50 per cent. or more of the patients will have ceased to be infectious by the time they are admitted. The two methods now commonly practised are the cough-plate and the postnasal swab.

Cough-plate : Points of importance for the success of the cough-plate method are .

(1) The medium should contain 30-50 per cent. of blood and the surface should be free from pitting (plates may be used up to two weeks after preparation provided they are kept in the cold and are free from moulds).

(2) Two plates should be exposed¹: for children of two years upwards 4-6 actual barks on to the plate are sufficient; for infants a longer exposure is required depending on the expulsiveness of the cough, but *over-exposure of the plate must be avoided* otherwise the colonies of *H. pertussis* are swamped by other more quickly growing organisms. Alternatively, the plates may be "treated" beforehand with penicillin (see below).

(3) Exposure of the plates during a natural spasm is usually recommended but requires speed, time and patience; in a busy ward or clinic an induced spasm, e.g. by pressure of the glottis—the use of a tongue depressor is apt to cause vomiting—gives satisfactory results

The inoculated plates may be sent through the post (for this purpose aluminium dishes are essential) or kept overnight in the cold without detriment. The plates are incubated at 37° C for three days, when they are examined, preferably with a hand or watch-maker's lens, for the tiny mercury-drop colonies of which in a sparsely inoculated plate there may be only one or two. Presumptive identification is made on morphology and slide-agglutination of a colony picked directly from the plate¹. Subculture on Bordet medium gives in 24-48 hours a good growth which has the appearance of aluminium paint. If the culture grows luxuriantly in 24 hours and produces blackening of the medium, it may be *B. parapertussis* which is an occasional cause of whooping-cough. Differentiation depends on the ability of *B. parapertussis* to grow on ordinary nutrient agar and on serological tests with specific (absorbed) antisera (Eldering and Kendrick, 1938).

Postnasal Swab: Disadvantages of the cough-plate method for the practitioner are the difficulties associated with the dispatch, satisfactory exposure and subsequent return of the plates to the laboratory. To overcome these difficulties various workers have used pharyngeal, laryngeal, or nasopharyngeal swabs (passed either through the anterior nares or through the mouth). The following method has given results at least as good as the cough-plate. The swabs can be taken by a nurse with an assistant to hold the child, and are then inoculated on to a plate of Bordet-Gengou medium containing penicillin (Cruickshank, 1944).

The swab is an ordinary metal throat-swab bent within $\frac{1}{2}$ in. of the end. With an assistant to hold the child's head and hands, and

¹ An agglutinating serum of good titre may be prepared in the rabbit by 3 to 4 one cc intravenous injections of a killed suspension of smooth *H. pertussis* (1,000 million organisms per cc.) at 5-7 day intervals.

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Acceptance of the isolation of *H. pertussis* from affected patients as diagnostic of whooping-cough assumes that this organism is the sole aetiological agent, and the following criteria in favour of such an assumption may be quoted .

(1) *H. pertussis*, a small gram-negative cocco-bacillus, has been isolated from over 90 per cent of patients in the early infectious stage of the disease and but rarely from convalescents or unaffected persons. (2) With pure cultures of the organism the infection has been reproduced in human volunteers and in apes, whereas this was not achieved with culture filtrates (3) Specific antibodies to *H. pertussis* develop in the blood of naturally or experimentally infected individuals , humans or other animals in which such antibodies are demonstrable after vaccination are resistant to attack. (4) Attempts to establish a filtrable virus as the cause of pertussis have failed.

For success in the isolation of *H. pertussis*, a reliable culture medium (see Appendix) and some experience in the recommended technique are necessary. It must also be remembered that in hospital practice, perhaps 50 per cent or more of the patients will have ceased to be infectious by the time they are admitted. The two methods now commonly practised are the cough-plate and the postnasal swab.

Cough-plate : Points of importance for the success of the cough-plate method are :

(1) The medium should contain 30-50 per cent. of blood and the surface should be free from pitting (plates may be used up to two weeks after preparation provided they are kept in the cold and are free from moulds)

(2) Two plates should be exposed¹: for children of two years upwards 4-6 actual barks on to the plate are sufficient; for infants a longer exposure is required depending on the expulsiveness of the cough, but *over-exposure of the plate must be avoided* otherwise the colonies of *H. pertussis* are swamped by other more quickly growing organisms. Alternatively, the plates may be "treated" beforehand with penicillin (see below).

(3) Exposure of the plates during a natural spasm is usually recommended but requires speed, time and patience; in a busy ward or clinic an induced spasm, e.g. by pressure of the glottis—the use of a tongue depressor is apt to cause vomiting—gives satisfactory results.

The inoculated plates may be sent through the post (for this purpose aluminium dishes are essential) or kept overnight in the cold without detriment. The plates are incubated at 37° C. for *three days*, when they are examined, preferably with a hand or watch-maker's lens, for the tiny mercury-drop colonies of which in a sparsely inoculated plate there may be only one or two. Presumptive identification is made on morphology and slide-agglutination of a colony picked directly from the plate.¹ Subculture on Bordet medium gives in 24-48 hours a good growth which has the appearance of aluminium paint. If the culture grows luxuriantly in 24 hours and produces blackening of the medium, it may be *B. parapertussis* which is an occasional cause of whooping-cough. Differentiation depends on the ability of *B. parapertussis* to grow on ordinary nutrient agar and on serological tests with specific (absorbed) antisera (Eldering and Kendrick, 1938).

Postnasal Swab: Disadvantages of the cough-plate method for the practitioner are the difficulties associated with the dispatch, satisfactory exposure and subsequent return of the plates to the laboratory. To overcome these difficulties various workers have used pharyngeal, laryngeal, or nasopharyngeal swabs (passed either through the anterior nares or through the mouth). The following method has given results at least as good as the cough-plate. The swabs can be taken by a nurse with an assistant to hold the child, and are then inoculated on to a plate of Bordet-Gengou medium containing penicillin (Cruickshank, 1944).

The swab is an ordinary metal throat-swab bent within $\frac{1}{2}$ in. of the end. With an assistant to hold the child's head and hands, and

¹ An agglutinating serum of good titre may be prepared in the rabbit by 3 to 4 one cc intravenous injections of a killed suspension of smooth *H. pertussis* (1,000 million organisms per cc.) at 5-7 day intervals.

using a tongue depressor, the doctor or nurse passes the tip of the swab up behind the uvula and rubs it on the posterior pharyngeal wall. The swab is then returned to the container, still slightly bent so that the inoculated portion does not rub against the side of the tube, and sent to the laboratory. If there is likely to be delay in reaching the laboratory 0.5 cc of 2 per cent. saline-agar should be placed in the bottom of the container and the top of the swab allowed to rest on the surface of the agar. This keeps the swab moist and prolongs the viability of the organism.

Each plate of the Bordet-Gengou medium is impregnated before use with 4 drops (≈ 0.13 cc.) of a suitable dilution of penicillin. The strength of penicillin solution required is judged by the inhibitory effect of different dilutions upon a number of test organisms—staphylococcus, haemolytic streptococcus, a diphtheroid organism, *M. catarrhalis* and *H. pertussis*—inoculated on the Bordet-Gengou medium. About 10–20 Oxford units are needed per plate of 12 cc. of medium. The weak dilution of penicillin in daily use tends to lose its potency, so that a fresh supply should be prepared weekly. The penicillin is added half to one hour before the plate is to be inoculated, the drops are spread evenly over the plate with a glass spreader and the plate is then dried in the incubator. The post-nasal swab is rubbed over about a third of the plate and a platinum loop is used *secundum artem* to inoculate the material over the rest of the plate (see Plate II).

Other Tests : Tests for the presence of specific antibody in the patient's serum—agglutination and complement fixation reactions—are useless for early diagnosis as they do not become positive until the third or fourth week of infection. A positive complement fixation test in a young child may, however, be used to confirm a retrospective diagnosis (Donald, 1938). So far intradermal tests with different pertussis fractions have not proved sufficiently specific to be used in diagnosis or as a test of susceptibility to the infection, although, recently, the use of a purified fraction (agglutigen) has given promising results (Flosdorf *et al.*, 1943).

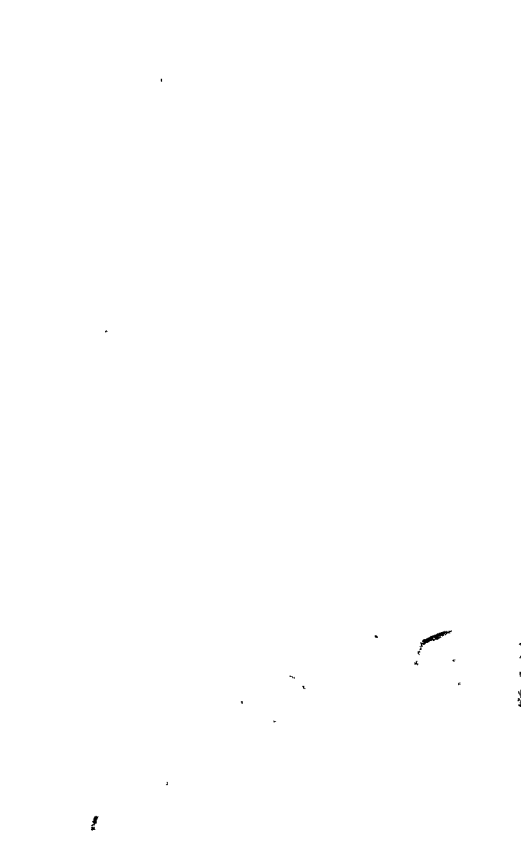
HAEMATOLOGICAL DIAGNOSIS

The total and differential white-cell count is not sufficiently used for the diagnosis of pertussis. The significant blood picture, particularly a relative lymphocytosis of 60 per cent. or more, may occur in the early catarrhal stage; in the second or third week of the disease, when the diagnosis may still be in doubt, a white-cell

PLATE II



H. pertussis on Bordet-Gengou medium post-nasal swab inoculated on penicillin treated plate (see text) The large colonies are resistant forms of *M. catarrhalis*.



count of 10,000 per cc. over the normal for the age and a lymphocytosis of 60-80 per cent. is in the presence of a suspicious cough usually diagnostic of pertussis. The lymphocytosis natural to young children must, of course, be kept in mind (the lymphocytes predominate until the fourth year of life) and the possibility of lymphatic leukaemia must be excluded. A blood examination is particularly helpful at clinics and outpatient departments or in private practice where a quick diagnosis is often so essential. If, in addition, it is possible to do an erythrocyte sedimentation-rate, the finding of a normal or retarded reading makes with the leucocytosis and suspicious cough a triad of symptoms practically pathognomonic of pertussis (Gold and Bell, 1936).

Appendix

Bordet-Gengou Medium. Clean and pare potatoes, cut into thin slices, add 500 g. slices to 500 cc. tap water. Add 9 g. sodium chloride and autoclave at 15 lb. for 30 minutes. While still hot mash with fork to pulp. Filter through gauze and make volume up to 2,000 cc. with water. Adjust pH to 7.0 with N/20 NaOH (only a very small volume of alkali is needed). Add 40 cc. glycerine and 20 g. peptone (Difco proteose or Evans' bacteriological peptone). Distribute in 50 cc. lots, using wide-bore pipettes, and keeping mixture well shaken during process of bottling. To each bottle add 2 g. agar-agar powder. Autoclave at 15 lb. for 15 minutes.

FOR USE: Melt potato-agar mixture, cool to 70° C. and add 50 cc. defibrinated horse blood, which should be warmed in the 37° C. incubator beforehand. Pour thick plates (approx. 6 plates per 100 cc.). The plates should not be dried in the incubator, but should be stored at once in the refrigerator, when they may be used up to two weeks after preparation.

R. CRUICKSHANK.

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CHAPTER VII PHYSIOLOGICAL DIAGNOSIS OF TUBERCULOSIS

... of tubercle bacilli
... diagnosis of tubercle
... diagnosis and
... bacteriological
... mass radiograph
... has been opened and
... technique has
... only the combination
... by reliable methods for
... will furnish the required result.
... of early and convalescent
... therapy or with fibrotic lesion
... unequal distribution of tubercle bacilli
... tissue fluids account for the fact
... repeated microscopical, cultural, and
... might in these cases give positive results.
It is therefore essential that laboratory
for the demonstration of tubercle bacilli

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the preparation of the tuberculous material,
with under the headings of: (a) Collection of
(c) Methods of concentration, (d) Culture media,
(a) Collection of Material. SPUTUM.

expectoration There is generally no difficulty in
right kind of sputum. Occasionally the specimen
extra-pulmonary nature obtained from the nasal
buccal secretion consisting of saliva. The patient
instructed that the morning cough usually provides a

It is advisable to collect the sputum specimen in
cardboard box which can be easily disposed of or in a
wide-mouthed glass container fitted with a screw cap.
containers can best be sterilised by autoclaving or by
containing caustic soda.

Patients with scanty expectoration, e.g., children

mental patients, patients on collapse-therapy. Three methods are used in order to overcome the difficulties in obtaining a suitable specimen: (1) Laryngeal Swab Culture; (2) Gastric Lavage; (3) Examination of the Faeces. The technique and respective merits of these methods are discussed later.

URINE: Either collection of a complete twenty-four hours' specimen of urine (this should be preserved by the addition of a little toluol), or collection of the morning specimen of urine only, on three consecutive days. In our experience the latter method has proved less laborious and as reliable as examination of the 24-hours' specimen.

CEREBROSPINAL FLUID AND EFFUSIONS: A portion of the specimen is collected into a clean dry sterile tube, while another portion is collected into a tube containing an anti-coagulant, e.g., sodium citrate.

PUS: collect into dry sterile tube.

FAECES: collect into sterile waxed cardboard container.

(b) Staining Methods. The tubercle bacillus does not stain with the ordinary dyes probably owing to its fat content. Ziehl-Neelsen's method overcomes this difficulty and is the method universally applied.

(1) Stain with Carbol-Fuchsin¹ for ten minutes, heating intermittently until stain is gently steaming.

(2) Wash off with water, flood with 25 per cent sulphuric acid (by weight in water) for five minutes.

(3) Wash well in water; film should now be pale pink.

(4) Counterstain one minute with 1 per cent. aqueous methylene blue or with 0.1 per cent malachite green (prepared from 1 per cent. malachite green in Aq. Dest.)

(c) Methods of Concentration. Methods of concentration are used either for the concentration of tubercle bacilli to improve the chances of microscopic examination, e.g. of sputum; or to destroy in specimens to be cultured for tubercle bacilli all secondary organisms which would outgrow the developing colonies of *M. tuberculosis*. The following methods are recommended.

(1) **PETROFF'S METHOD.** This is nearly as effective as the old antiformin treatment in breaking down masses of mucus, etc.

Basic Fuchsin	.	.	.	1 g.
Abs. alcohol	.	.	.	10 cc.
Solution of carbolic acid (1:20)	.	.	.	100 cc.

Dissolve the dye in the alcohol and add the solution to the carbolic acid

CHAPTER VII

BACTERIOLOGICAL DIAGNOSIS OF TUBERCULOSIS

THE demonstration of tubercle bacilli remains the most conclusive evidence in the diagnosis of tuberculosis. The most important consideration in the diagnosis and control of pulmonary tuberculosis revolves on the bacteriological findings in the sputum. With the introduction of mass radiography a vast new field for laboratory investigation has been opened and the importance of accurate and reliable laboratory technique has become even more evident. In many instances only the combination of several methods or repeated examination by reliable methods for the detection of the tubercle bacillus will furnish the required result. The scarcity of organisms in the sputum of early and convalescent cases, in patients on collapse-therapy or with fibrotic lesions, and in caseous matter, and the unequal distribution of tubercle bacilli in sputum, urine and other tissue fluids account for the failure of a single examination. Repeated microscopical, cultural, and/or animal inoculation tests might in these cases give positive results.

It is therefore essential that laboratory methods and technique for the demonstration of tubercle bacilli should be given careful attention by laboratory workers, and this chapter deals with various aspects of the subject in detailed form.

The bacteriological diagnosis of tuberculosis depends largely upon the preparation of the tuberculous material. This can be dealt with under the headings of : (a) Collection of material, (b) Staining, (c) Methods of concentration, (d) Culture media, (e) Care of animals.

(a) Collection of Material. SPUTUM: Patients with good expectoration. There is generally no difficulty in obtaining the right kind of sputum. Occasionally the specimen received is of an extra-pulmonary nature obtained from the nasopharynx or merely buccal secretion consisting of saliva. The patient must be instructed that the morning cough usually provides a suitable specimen.

It is advisable to collect the sputum specimen in either a waxed cardboard box, which can be easily disposed of by burning, or a wide-mouthed glass container fitted with a screw cap. The glass containers can best be sterilised by autoclaving or boiling in water containing caustic soda.

Patients with scanty expectoration, *e.g.*, children, female and

deposit is less concentrated than after alkali treatment and is therefore less suitable for microscopy

Concentration methods ought to be carried out with great care and intelligence, with due regard to the value of the specimen. All recommended methods have probably some toxic effect on the tubercle bacillus. They should not, therefore, be used for urine, pus, C.S.F. etc, where there are no contaminating non-acid-fast organisms

Most workers advise the use of new slides for smears. Old slides can be used according to Hunter in this way. Put used slides into benzol to dissolve the oil. Transfer into methylated spirit for a few minutes, then into water. When a number of slides have accumulated they are boiled for two hours in soapy water (soft soap), allowed to cool, and then each slide is washed in running water. Immerse in cleaning solution (Pot. bichromate 60 g., conc. sulphuric acid 60 cc, water 940 cc.) for at least 24 hours; wash in hot water thoroughly and allow to dry. Inspect and discard any slide with a blemish.

Fluorescence microscopy should be mentioned, as it appears to be the only microscopical method so far superior to Ziehl-Neelsen's method, but it requires special apparatus. A relatively inexpensive and apparently reliable technique has recently been described (Lempert, 1944).

(d) **Culture Media.** The cultivation of *M. tuberculosis* is not difficult provided that good media are used and the method of concentration is carried out with care. The material is transferred either directly, e.g. C.S.F., or if contaminated a concentration method has to be applied before inoculation.

The first to use inspissated eggs for the cultivation of tubercle bacilli was Dorset in 1902. In recent years many egg media have been published aiming at improving the nutrient value of the medium and checking secondary organisms. Three methods of the preparation of reliable egg media are given :

(1) **DORSET'S EGG MEDIUM** (after Mackie and McCartney)

Break into a sterile bowl fresh eggs which have been washed in soap and water and then dried, beat thoroughly with a sterile knife to mix the yolks and whites. Strain the mixture through sterile cheese-cloth over a filter funnel, and to every 75 cc. of egg mixture (usually two eggs) add 25 cc. of sterile digest broth¹ and 1 cc. of a 1 per cent aqueous solution of crystal violet. Tube the medium in small sterile bottles

¹ Some workers prefer the medium without digest broth according to the original Dorset formula.

and has the additional advantage of being less lethal to the tubercle bacilli than antiformin.

Technique · Sputum is mixed thoroughly with three to four times its volume of 4 per cent caustic soda and placed in the incubator at 37° C. for 10–30 minutes¹, the container being shaken from time to time. The mixture is centrifuged at 3000 r.p.m. and the supernatant fluid poured off into a lysol jar. The deposit is neutralized with 8 per cent. hydrochloric acid which is added drop by drop, the reaction of the mixture being tested by touching litmus paper with a small loopful (the litmus paper is placed in a sterile Petri dish and small drops of the mixture tested with a sterile loop). The deposit is stained, cultured or inoculated into animals.

The neutralization can be determined also by adding a drop of phenol red to the deposit.

(2) **JUNGMAUN'S METHOD** (modified): This method is based upon the liquefaction of the sputum in distinction to homogenization as in other methods.

Two solutions are needed ·

Solution A :

Ferrous sulphate	20 g
Conc sulphuric acid	20 cc
Distilled water	180 cc

Solution B :

Hydrogen peroxide	1 vol. per cent.
(5 parts of 20 Vol. H ₂ O ₂ : 95 parts water)	

Solution A can be made up in bulk and kept indefinitely.

Solution B must be made up freshly on each occasion.

Technique 6 cc of a mixture of equal parts of solutions A and B are added to 5 cc. of sputum. On standing for 10–15 minutes the solution of mucus is complete.

After centrifugation the deposit is washed once or twice with normal saline or neutralized with N/1 NaOH.

It was originally recommended to neutralize the deposit by washing with 5 % sodium citrate or 2 % sodium lactate, but these solutions readily become contaminated.

The main advantages of Jungmann's method are speed and effective elimination of secondary organisms without affecting the staining properties and the viability of the tubercle bacilli. The

¹ The duration of treatment with alkali will depend on the specimen. If thin and watery 10 minutes is long enough : if muco-purulent 30 minutes treatment is usually safe.

deposit is less concentrated than after alkali treatment and is therefore less suitable for microscopy.

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¹ Some workers prefer the medium without digest broth according to the original Dorset formula.

(avoiding the formation of air bubbles) and coagulate in a slightly sloped position in the inspissator at 75° C. Sterilize by heating in the inspissator at 75° C. for two hours the next day.

This medium is particularly useful for the cultivation of the bovine type and dysgonic human strains of the tubercle bacillus.

(2) LÖWENSTEIN-JENSEN'S MEDIUM

(after Mackie and MacCannell)

(i) Mineral Salt Solution. Chemically pure salts must be used

	per cent.
Potassium dihydrogen phosphate KH_2PO_4 (Analar)	0.4
Magnesium sulphate (Analar)	0.04
Magnesium citrate	0.4
Asparagin	0.6
Glycerol (Analar)	2.0
in distilled water.	

600 cc is a convenient quantity to prepare. Heat to dissolve. The solution is boiled or placed in the steamer for two hours and allowed to cool overnight. Jensen stresses the importance of the particular concentration of glycerol in the medium.

(ii) Salt-Starch Solution. To each 600 cc. are now added 30 g. of potato starch. This is mixed and heated in a water-bath with constant stirring for 15-20 minutes until a satisfactory paste is produced and then allowed to remain for one hour in a water-bath at 56° C.

(iii) Egg Fluid. Newly laid hen's eggs are washed thoroughly in a 5 per cent. soft soap solution after which they are left in running water for one to two hours. The eggs must be less than one week old and 20 eggs are usually sufficient for 1 litre of egg fluid, but if they are small, 22 eggs should be used. The eggs are opened at both ends with a sterile knife and held over a sterile funnel containing a gauze filter through which they are filtered into a sterile vessel.

For each 600 cc. of salt-starch solution, 1 litre of egg fluid is required. The egg fluid can also be first mixed very thoroughly and then filtered. To the egg filtrate is added the salt-starch solution at the termination of one hour in the water-bath at 56° C.

(iv) Malachite Green Solution. Make a 2 per cent. solution of malachite green in distilled water and place in the incubator for one to two hours. To each 1,600 cc. of prepared substance (1 litre egg fluid plus 600 cc. salt-starch solution) add 20 cc. of 2 per cent. malachite green.

The medium is now tubed in 5 cc. amounts in 1 oz. bottles and the caps tightly screwed on. The bottles are laid horizontally in the inspissator and are heated at 75° C. for 2 hours. They are to remain there overnight and are heated for 1 hour.

On this medium the human type of tubercle bacillus grows luxuriantly in the form of large

They are
hour.

Thus
y 7.

colonies, while the bovine type shows small discrete colourless colonies. Some authors use the medium with and without glycerol simultaneously in order to improve the chances of isolating the bovine type.

The addition of charcoal is claimed by Nassau to have improved cultural conditions by shortening the time necessary for the development of primary cultures, but we have not been able to confirm this claim.

(3) PETRAGNANI'S MEDIUM

To 360 cc. of fresh milk add
15 g. of potato meal (B.D.H.)
3 small finely grated potatoes

Place in a beaker in a water-bath at 100° C. stirring constantly for 10 minutes. Then allow the beaker to remain in the water-bath covered with a lid for another hour until a homogeneous thick pasty consistency is reached. Remove the mixture and allow to cool to 60° C.

Add 12 whole eggs prepared as above, then 30 cc of glycerol and 20 cc of 2 per cent aqueous malachite green. Mix well, tube and coagulate at 75° C for one hour. Sterilize by inspissation at 75° C. for 20 minutes on two to three successive days.

The media tubes are best removed from the inspissator overnight. This allows the formation of sufficient water of condensation.

Media for Rapid Identification. Even on the best media the time necessary for the diagnostic culture of tubercle bacilli is comparatively long. As any method of shortening this time is of practical value, we include two methods published in recent years which reduce the average incubation period of cultures of the tubercle bacillus from two-three weeks to one week.

(i) Pryce's Slide Culture method.

(ii) Soltys-Friedmann Embryonic Tissue medium.

(i) Pryce (1941) described a method of growing tubercle bacilli in sputum films in which results were obtained in seven days or less. The films were made on a glass surface, treated with acid to kill contaminants, washed in sterile water to remove the acid and incubated in a culture medium consisting of equal parts of citrated blood and 1 per cent. saponin solution. After seven days the films were washed, dried and stained. Characteristic colonies developed during the time of incubation. The method was applied in clinical work by Rosenberg, who cultured the slide cultures in Kirchner's fluid medium for one week. Clumps of tubercle bacilli could usually be demonstrated with the 2/3 objective of the microscope,

after one week of incubation. Care should be taken to avoid contamination.

(ii) Embryonic Tissue medium. Soltys (1942) used a fluid medium containing chick embryonic tissue (2 per cent.) in Tyrode's solution for the study of type cultures of *M. tuberculosis* and described excellent growth after 3-7 days. Friedmann (1944) in the course of experiments on silicosis used a similar medium and applied it for clinical purposes. In order to reduce the risk of contamination, penicillin was added to the medium.

MEDIUM. To 25 cc. of Tyrode's solution in conical flasks of 50 cc. capacity 0.5-1 cc. of chick embryo pulp is added. Embryonic pulp is prepared by mincing finely a chick embryo 11-12 days old. Penicillin is then added (300-500 Oxford units).

Primary cultures were successfully grown from tuberculous sputa, urines and C.S.F. on this medium.

The inoculum is prepared in the usual way. Sputum is treated with 6 per cent. sulphuric acid and washed without neutralisation. 0.5-1.0 cc. or more of the washed deposit can be inoculated.

Smears are made by removing fragments from the flasks and squashing them on slides. Often already after 3-5 days large bundles of tubercle bacilli are found lying freely or within the tissue. Later serpiginous colonies appear forming Medusa-head formations.

The Medusa-head colony prevails in cultures of the human type of *M. tuberculosis*. The bovine type shows a tendency towards the formation of more compact nest-like colonies. The avian type grows loosely or in small groups of single bacilli often around or in macrophages.

Care of Animals. The scope of this article does not allow the detailed discussion of this problem. The condition of test animals has a considerable bearing upon the ultimate result of the test. Intercurrent diseases may often interrupt the course of experiments. These can often be traced to latently infected animals, unsuitable animal quarters, careless attendance and feeding. The care of the animals is often left to untrained and unreliable personnel. Proper animal quarters under the attendance of skilled personnel are essential for successful results from animal inoculation.

The Examination of Sputum

Smears. The sputum is carefully searched for purulent particles. It may be advisable to transfer it from the collecting vessel

into a sterile Petri dish placed on black paper. A suitable portion is picked out, preferably with a Pasteur pipette, sterile forceps or a wooden splinter and transferred to a slide. A thin uniform film can best be obtained by warming the slide in the Bunsen flame. Dry the film gradually in the incubator. The film is stained by the Ziehl-Neelsen method and then carefully examined for 10 minutes. A $1/7$ oil-immersion lens can be recommended, and a green filter placed between the microscope and a good illuminant is helpful, particularly for the colour-blind person. It should be noted that in films stained by Ziehl-Neelsen's method red-stained organisms in the midst of hyaline material must not be regarded as tubercle bacilli as such material may resist decolorization. Two or three separate acid-fast bacilli should be seen before a positive report is given.

The report should give the result on the finding of tubercle bacilli and describe the cytology of the specimen. A negative result in a mucoid sputum is of much less significance than a similar result in a muco-purulent or purulent specimen, which should always be repeated. In purulent sputums a direct film will usually reveal the bacilli without further concentration. Specimens, however, with little pus or of gelatinous texture may be advantageously examined by one of the concentration methods.

Culture. The alkaline concentration methods are satisfactory for direct microscopy, but the sulphuric acid method is preferable for cultures. Cultures are grown on Lowenstein or Petragnani medium containing glycerol as almost all strains belong to the human type. The cultures are examined each week for growth, in particular after the first week for possible contamination, in which case they are discarded. Growth usually occurs after 10-20 days, an occasional strain needing longer to develop macroscopic colonies. If a culture is negative after four weeks it is unlikely that growth will ensue but positive cultures occasionally appear as late as six weeks.

The characteristic colonies of the human type can be easily recognized. Occasionally a smooth variant may be seen with entire edge slightly raised only and less pigmented. When in doubt a smear should be made and the acid-fast nature and the difficulty with which the colony is broken up, noted. The colony of a saprophytic acid-fast organism emulsifies easily and the bacilli are

orange red or bright shiny yellow colony of the saprophytic organism.

GASTRIC LAVAGE. First described by Poulsen the bacteriological examination of stomach contents has proved its value in the hands of many investigators. It is especially used in the investigation of childhood tuberculosis. But it has been more generally applied in all patients who fail to produce sputum.

Technique the gastric content should be collected in the morning before breakfast. A sterile gastric tube (Ryle's tube) is used, and 4 ozs. of sterile water may be injected through the tube into the stomach. After about 10 minutes the content is aspirated with a sterile syringe and put into a sterile bottle or cotton-wool-plugged Erlenmeyer flask. In the laboratory the specimen may be allowed to settle for 4-6 hours in the ice-chest¹. After decanting the supernatant fluid the loose deposit is centrifuged and a direct smear made and stained. The deposit is then treated with 6 per cent sulphuric acid and cultured. In specimens received from children, Dorset's medium should also be inoculated.

The result of the direct smear alone should not be taken as proof of activity since saprophytic acid-fast bacilli may be present. In institutions there is usually no urgency for a quick result and direct smears can be dispensed with. In order to avoid mistakes the collecting vessels must after use be put into strong lysol (50 per cent.) for several hours and thoroughly rinsed afterwards. Gastric lavage is a most valuable method in hospitalized patients but is less practicable in out-patients or large-scale investigations. The laryngeal swab may prove to be a more suitable method for such cases.

LARYNGEAL SWAB A strong flexible wire, chromium nickel, is bent two inches from one end (whole length nine inches) and the bent end is wrapped firmly with cotton wool. The wire of post-nasal swabs can also be used. The wire swab is placed in a tube plugged with cotton wool and sterilized, or a large number of swabs can be sterilized in a tin container. Before use the wire is dipped into sterile water as sputum adheres better to the moist surface. The swab is passed down the larynx in front of the vocal cords either under direct vision or by using a laryngeal mirror. The wire is withdrawn and straightened by sterile forceps (sterilized by flaming), returned to the tube and sent to the laboratory. Preferably two swabs are taken because the material collected on the swabs is rather scanty. The swabs should reach the laboratory and be inoculated on culture media as speedily as possible. Six per cent sulphuric acid is poured into the tube which is incubated for 5-10 minutes. The acid is then poured off and replaced by 1 cc of 4 per cent sodium hydroxide. A few seconds will suffice to neutralize the acid on the swab. Two tubes of a suitable medium are inoculated from each swab by rubbing all sides of the cotton wool swab on the

¹ Specimens must not be allowed to stand at room temperature for any length of time as the gastric juices may destroy the tubercle bacilli.

surface of the medium. If any mucus is seen floating in the acid it should be recovered by centrifuging; the deposit should be neutralized and cultured. Direct smear examinations use up the very scanty material and are therefore not recommended.

The cultures are incubated for 6 weeks. The procedure is simple and can be repeated on two or more consecutive days. The laryngeal swab culture has proved its value in mass investigations, and also in clinical patients who have no sputum or swallow their sputum or who become sputum-free following the induction of artificial pneumothorax. The assessment of treatment and the differential diagnosis of radiological opacities have been greatly assisted by the use of laryngeal swab cultures.

Pleural Effusion

Tuberculous pleural effusions by their appearance can be classed as clear, turbid or purulent, with gradations from one to the other. From the laboratory point of view they exhibit slightly different properties and their examination will be dealt with separately.

CLEAR EFFUSIONS *Cytology*: White blood cells are not numerous; the majority are lymphocytes. A few red blood corpuscles are invariably present and an occasional large cell of the histiocyte type. A few polymorphs may be found but only rarely do they exceed 50 per cent of the total. Occasionally a large number of eosinophiles are present in the early stages of the effusion. Some fibrinogen is present and clotting occurs frequently.

Specific gravity and protein content give little help in determining the aetiology of the effusion.

The microscopic detection of tubercle bacilli in serous effusions is rarely possible. In *primary tuberculosis* the pleura reacts to the formation of the primary focus which as a rule lies immediately beneath it. A small nodular spread in the neighbourhood of the primary focus is fairly common and is bound to involve the pleural cover. In *disseminated tuberculosis* blood-borne bacilli may reach the pleura without affecting the lungs, causing the formation of tubercles or diffuse caseation. In other cases the pleurisy may be a reaction to an intrapulmonary spread without specific tubercular tissue changes on the pleura itself. The whole process is not directly elicited by organisms and conveys the impression of allergic hypersensitivity of the pleura, so that absence of tubercle bacilli in the pleural fluid does not militate against the tuberculous origin of pleurisy.

However, many investigators are of the opinion that any effusion

due to tuberculosis is caused by a definite infection of the pleura with tubercle bacilli. Since the total number of organisms is small, it is obvious that in using small quantities of clear effusion the few bacilli dispersed in the whole volume can easily be missed when culture or guinea-pig inoculation is attempted. By using larger quantities, more positive cultures can be obtained than with small quantities as used by many workers.

Culture collect as large a quantity of the effusion as the condition of the patient will permit into a sterile flask. About 20 cc. should also be collected into a tube containing a suitable anti-coagulant, e.g. a 1-oz. screw-capped bottle containing four drops of 20 per cent sodium citrate solution. The main specimen is cultured on blood-agar for secondary organisms and the flask kept overnight in the ice-chest. The specimen with the anti-coagulant is centrifuged and two films are made and stained with Leishman and Ziehl-Neelsen. If many polymorphs are present stain with Gram.

After 24 hours examine the fluid in the flask. If a clot has formed transfer it into a sterile Petri dish. Part of the clot is detached, spread on a slide, dried slowly and re-spread just before complete desiccation. This film is stained with Ziehl-Neelsen, although, as already noted, tubercle bacilli are rarely, if ever, found in cellular deposits or fibrinous clots of clear serous effusions.

If the blood agar culture is sterile, no treatment with acid is necessary. The rest of the clot is used, rubbing it well over the surface of the medium and leaving the clot well spread on it. Cultures are also made from the centrifuged deposit of the specimen containing anti-coagulant.

TURBID FLUIDS *Cytology* a greater number of polymorphs are present and the protein content is higher. These two factors account for the turbidity of the fluid.

Technique: a smaller amount collected into a bottle containing sodium citrate is usually sufficient. A blood agar plate is inoculated for the presence of secondary organisms and the specimen is kept in the ice-chest overnight. The centrifuged deposit is then treated in the same way as outlined above.

PURULENT FLUIDS: in specimens from tuberculous empyemas no centrifuging is required. Otherwise follow the technique as outlined above. In cases where previous investigations have yielded negative results it is useful to digest the pus with alkali and culture the more concentrated specimen.

Various investigators have sometimes attached particular significance to one or more of the findings in pleural effusions in relation

to prognosis and treatment. It is very difficult to say from one fluid examination what the prognosis and development of the effusion will be except in purulent effusions where the prognosis is usually grave. In clear effusions which on previous examination were of the lymphocytic type the appearance of polymorphs is of significance. A number of these cases become empyematous. Sugar estimations have been claimed to have prognostic value, but one of us (K S R) in a series of cases could not confirm this finding.

Urine

The importance of the detection of tubercle bacilli in the urine cannot be exaggerated. The examination of urine specimens for tuberculosis must be conducted with great care and patience. Frequently there are only a few organisms present, or the organisms may be excreted intermittently.

Collection of the Specimen. The usual method is the collection of a complete 24-hours' specimen of urine (preserved by the addition of 3-5 cc. toluol). The specimen is allowed to stand in the ice-chest overnight. The supernatant fluid is carefully poured off and the remainder spun down for half an hour. This method is satisfactory if carried out by trained personnel. But too often the specimen is left standing in the warm ward, is delivered late and is profusely contaminated with spore-bearing organisms, etc.

Examination of the morning specimen of urine is a suitable alternative procedure. It is left standing for a few hours and then centrifuged. The whole deposit is collected, stained and cultured. This method can be carried out on three consecutive days.

Direct smear: the deposit is stained with Ziehl-Neelsen. The specimen should be decolorized with 96 per cent. alcohol in addition to the usual decolorization in acid, as smegma bacilli, short and plump, are said to resist acid but not alcohol decolorization.

Tubercle bacilli are irregularly distributed, and commonly a small group is found only after prolonged search of the whole smear. It is very unusual to find tubercle bacilli in the absence of pus. All urines on the other hand containing pus and proving sterile on culture, also urines containing pus and growing *Staphylococcus albus* or *aureus*, should be examined for tubercle bacilli. A diagnosis of a tuberculous urinary infection should preferably not be made on a direct film examination unless corroborated by a positive culture. Guinea-pig inoculation is given preference by some workers.

Culture: the deposit is treated with 6 per cent. sulphuric acid or 4 per cent. NaOH and cultured on both glycerinated and non-glycerinated egg media. If the deposit contains masses of contaminants, half an hour's treatment with acid or alkali is not always sufficient to kill all secondary organisms and contamination of cultures may ensue. It is therefore advisable to treat two lots of deposits, one in the usual manner, the other for a slightly longer period (controls of sterility can be made on agar while the deposit is kept in the ice-chest).

Cerebrospinal Fluid

The detection of tubercle bacilli in the direct smear is diagnostic. The cytological and chemical examination of the C.S.F. may support the diagnosis

Characteristics of the C.S.F. in tuberculous meningitis:

Appearance: opalescent or slightly turbid.

Cytology: 20-100 lymphocytes and a few polymorphs per c.mm

Chemistry. Protein: increased.

Globulin. increased.

Sugar. normal or reduced.

Chlorides: reduced.

Spider-web clot forms on standing

Tubercle bacilli in clot.

There are many exceptions to this classical description. Thus in early cases and young children the response is often polymorphonuclear at first and may remain so in children throughout the whole disease. The cell count is commonly below 100 but may be higher. On occasions very low cell counts of 20 or so may be found. The excretion of tubercle bacilli is intermittent and repeated punctures may be necessary to find them. The clot formation is not constant. The centrifuged deposit can be used. Chlorides may not be significantly reduced in the early stages of the infection

TECHNIQUE: After the cell-count, which is done at once, the specimen of C.S.F., if it is clear or opalescent, should be left standing to enhance a good cobweb formation; such cobwebs form often within a few hours. After the clot has been fished out, the specimen is centrifuged and the deposit is used direct for TB smear and cultures. The supernatant fluid can be used for biochemical examinations.

Examination of the spider-web clot: place the clot on a slide, spread out with the help of a needle and dry in incubator. Spread out again before the clot has completely dried.

Clear C.S.F. sometimes does not form any clot. There are only very few cells present in the scanty deposit or no deposit is obtained on centrifugation. Yet it is advisable to culture the last few drops remaining at the bottom of the tube. We have seen positive cultures develop from these apparently "negative" fluids.

Pus

Specimens of pus vary according to their source. Pus from a tuberculous empyema usually shows a great number of tubercle bacilli (see "Pleural Effusions"). Aspiration pus from glands contains very few bacilli. The appearance of pus from glands is often typical, being of a thick cheesy consistency and greyish yellow colour. As a rule there are no secondary organisms present. Microscopic examination shows disintegrated cell matter and an occasional polymorphonuclear white cell or fibrocyte. Pus from glands should invariably be inoculated on both glycerinated and non-glycerinated egg media, since the infection is often due to the bovine type.

Faeces

The examination of faeces may be required for two reasons: (1) The patient swallows his sputum or does not expectorate. (2) In the diagnosis of tuberculosis of the intestines (if excretion of tubercle bacilli from the lungs can be excluded).

TECHNIQUE. Equal volumes of faeces and 4 per cent. sodium hydroxide are mixed and thoroughly shaken. The mixture is incubated and inspected from time to time. When gross organic matter has disappeared and the mixture has taken on a uniform yellow appearance, it is neutralized with 4 per cent. hydrochloric acid drop by drop in the presence of Bromthymol blue. The material is then centrifuged and the deposit stained and cultured. Acid-fast bacilli in faecal smears cannot be accepted as tubercle bacilli without confirmation by culture or animal inoculation. But cultures are apt to be contaminated by the growth of sporing organisms, while guinea-pigs inoculated with faeces frequently die within a few days of *Cl. welchii* infection.

Animal Inoculation

Modern cultivation methods compare favourably with animal inoculation but use of both methods counterbalances certain inherent disadvantages in each. As a routine, a combination of both methods

will permit a more accurate detection of tubercle bacilli present in small numbers than will either method alone.

The table shows the results obtained by K. E. Jensen (*Tuberculosis Memo*, 1936).

COMPARISON OF CULTURE ON LÖWENSTEIN-JENSEN MEDIUM AND GUINEA-PIG INOCULATION FOR ISOLATION OF TUBERCLE BACILLUS FROM MICROSCOPICALLY NEGATIVE MATERIAL
(Jensen ; see Memo, Joint Tuberc. Coun., 1936)

Material	Combined methods H + B		Culture				Inoculation			
			H		B		H		B	
			+	-	+	-	+	-	+	-
Sputum and gastric lavage	717	52	649	68	43	9	580	137	44	8
Pleural exudate	89	8	73	16	6	2	64	25	6	2
Urine	233	50	205	28	34	16	221	12	49	1
Pus and tissue	135	63	131	4	54	9	118	17	56	7
Spinal fluid	142	36	139	3	31	5	123	19	32	4
TOTALS :	1316	209	1197	119	168	41	1106	210	187	22

H = Human type

B = Bovine type

Technique of Guinea-pig Inoculation : Two healthy guinea-pigs of 300-450 g. weight are used. 0.5-1 cc. of the material is injected subcutaneously into the flank or intramuscularly into the thigh. The pathological material can be used untreated or "treated" against secondary organisms. In the latter case, the deposit should be neutralized before injection. Thick pus should be emulsified in sterile saline to make injection of it possible.

The animals are examined 2-3 weeks after inoculation and then at weekly intervals, watching the inguinal glands. The animals are painlessly killed after six weeks and autopsied.

A fully virulent strain of human or bovine type will produce typical lesions in the guinea-pig: swelling and caseation of the regional lymph glands and of the glands further along the lymph passage; viz. the inguinal, lumbar and coeliac glands. The spleen invariably shows nodules and so does the liver. The lungs may be the seat of small tubercles, sometimes of caseous lesions. The

kidneys remain free. Smears from a caseous gland and from the spleen are stained for tubercle bacilli. Direct smears from at least two different organs should be examined for the presence of tubercle bacilli. Should the lesions be confined to the liver and the spleen, not involving the glands, pseudo-tuberculosis or *Salmonella* infection should be considered. In doubtful cases, material should be cultured or inoculated into a fresh guinea-pig. (For the guinea-pig lesions following the injection of *Br abortus*-infected milk, see p 64)

I. FRIEDMANN.

K. S. RODAN.

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CHAPTER VIII

LABORATORY DIAGNOSIS OF BRUCELLA INFECTIONS

It is now widely realised that the possibility of *Brucella* infection must be borne in mind in all pyrexias of unknown origin. The complete laboratory diagnosis of undulant fever includes the isolation and typing of the infecting strain whenever possible, and the demonstration of agglutinins in the patient's blood.

IDENTIFICATION OF BRUCELLA TYPES

The methods used for differentiating the bovine, caprine and porcine types of *Brucella* may be considered first, although of course in Great Britain *Br. abortus* is the only infecting type likely to be met. *Br. abortus*, *Br. melitensis* and *Br. suis* are culturally and biochemically very similar and agglutinate any *Brucella* serum almost to the same titre. Limitations of space do not allow full discussion of the various methods used in the differentiation of the three types (see Topley & Wilson, 1946, Chapter 34). The following table gives the chief differential criteria :

Type	Growth in absence of added CO ₂	Growth in presence of Thionin	Growth in presence of Basic Fuchsin	Production of H ₂ S	Antigenically (by agglutination with monospecific sera).
Melitensis	+	+	+	-	Melitensis
Abortus	-	-	+	+	Abortus
Suis	+	+	-	+	Abortus

For these tests, strains may be grown on 5 per cent. serum agar. In practice, the serum agar slope cultures required for the various tests are put up in duplicate and one set is incubated aerobically, the other in 10 per cent CO₂; about six slopes in all usually suffice.

Production of H₂S is tested for with lead acetate paper. *Br. melitensis* may produce a small trace in the first 24 hours, e.g. about 1 mm. of blackening. *Br. abortus* produces a considerable amount in the first two days, e.g. about 10 mm. on the second day, and then tails off, while *Br. suis* continues to produce H₂S for four days.

A valuable test introduced by Huddleson depends on the differential growth of the three *Brucella* types in the presence of certain

dyes, principally thionin and basic fuchsin. The method is valuable for all *Brucella* strains and is especially useful in differentiating between *Br. abortus* and *Br. suis*. Standard dyes, however, must be used and the preparations of satisfactory dye plates is not easy without some experience. (For the precise technique of the method see Huddleson, 1929)

Serology. The principles involved in the serological examination of *Brucella* strains with monospecific sera may be briefly described:

(1) All strains used in the preparation of specific sera and in agglutination tests must be in the smooth phase. Smoothness may be tested by the thermoagglutination method as follows:

The growth from a 48-hour serum agar slope is carefully emulsified into 2-3 cc. of normal saline, and 1 cc. of this dense suspension is immersed for two hours in a beaker of boiling water. A smooth strain should remain in perfect suspension during this time while rough strains show agglutination of varying degrees even after five minutes' immersion.

(2) Straight agglutinating sera are readily prepared in rabbits by intravenous inoculation of killed *Brucella* suspensions washed off in 0.25 per cent. formal saline and heated to 55° C. for half an hour. The initial dose is about 500 million organisms, and by inoculating doubling doses at three-day intervals one can usually have a serum of good titre by the end of three weeks.

(3) Empirically it is found that if an *abortus* serum has a titre of say 1/2400 (which will of course agglutinate *Br. melitensis* to almost the same titre), then this serum diluted one thirtieth to one sixtieth of its titre, i.e. about 1/40 to 1/80 in this case, if absorbed with an equal volume of a 3,000 million per cc. suspension of *Br. melitensis* will yield a monospecific *abortus* serum. The absorption can be done in about three hours at 37° C. The optimal absorbing dose is determined by a few preliminary absorptions at various dilutions of serum. If the absorbing dose of the heterologous strain is too small, the absorbed serum will not be monospecific; if on the other hand it is too large the titre of the absorbed serum even to *Br. abortus* is too low to be of use.

(4) Formalised heat killed suspensions of *Brucella* strains which are being typed are put up for agglutination against monospecific *abortus* and *melitensis* sera in final dilutions of 1/4 to 1/32. An *abortus* strain will usually be agglutinated to 1/16 or 1/32 by a monospecific *abortus* serum and not at all by monospecific *melitensis* serum.

DIAGNOSIS OF HUMAN INFECTIONS

In the diagnosis of *Brucella* infection in man, two principal methods are used, viz. blood culture and the agglutination reaction.

(1) **Blood Culture.** Early work in Malta showed that blood culture was positive in 80 per cent. of *melitensis* infections. It is most likely to be positive if blood be taken during the rise of the pyrexial wave. Most cases of undulant fever have a viable count of less than ten organisms per cc. of blood, so that a considerable volume of blood should be added to the culture-medium.

In contrast with the high percentage of blood culture isolations from *melitensis* and also from *suis* infections, only 10-20 per cent. of *abortus* infections reported in this country have had positive blood cultures. Undoubtedly failure to isolate *Br. abortus* from the blood in the past has been at least partly due to lack of appreciation of the need for increased CO₂ tension, but another factor may be the mildness of many *abortus* infections and the consequent absence of bacteraemia.

The technique of blood culture in general use nowadays is to inoculate 5-10 cc. quantities of blood into various media, such as 2 per cent. glucose broth, 5 per cent. serum broth, and Huddleson's liver broth. Some are incubated under ordinary aerobic conditions and some in 10 per cent. CO₂. Growth of *Brucella* cannot be judged by direct films but requires subculture at 3-5 day intervals on to appropriate solid media which must be, of course, in turn incubated under suitable atmospheric conditions. Original cultures are kept going for at least a month before being discarded.

As the growth of *Brucella* is often very slow it is important to avoid contamination of the blood cultures during the initial manipulations and later subcultures. Some workers suggest the incorporation of 1 in 500,000 gentian violet into one of the culture tubes to inhibit the growth of contaminants. Contaminating moulds may grow on the surface of blood cultures even when gentian violet is used. West and Borman find that mould contamination can be avoided by the use of screwcap bottles, instead of cotton-wool plugs, and the introduction into the bottom of the CO₂ jar of calcium chloride as a desiccating agent.

A small proportion of cases, variously reported as from 1 per cent. to 6 per cent., never develop blood agglutinins and for this reason it is desirable to attempt the isolation of the organism in all suspected cases. Further, positive blood culture is the only final evidence of active infection.

As an alternative to culture in artificial media, isolation of *Brucella* from the blood may be attempted by means of animal inoculation, as is customary with milk specimens.

A guinea-pig inoculated intramuscularly in a hind leg with *Brucella*-infected material and killed after six weeks shows enlargement of femoral and sublumbar lymph nodes, a spleen moderately enlarged, with an irregular surface and a few small grey necrotic foci and usually a few very small foci of necrosis on the surface of the liver. Cultures are readily obtained from the spleen and sublumbar lymph nodes and identified by the methods already described. The spleen of a *Brucella*-infected guinea-pig may resemble that of tuberculosis, but otherwise the differentiation of the two conditions is easy, tuberculous guinea-pigs show a caseating local lesion at the site of inoculation, widespread enlargement and caseation of regional and distant lymphnodes, gelatinous tubercles in the lungs, and, microscopically, acid-fast bacilli are seen in films of the caseous material.

(2) **The Agglutination Reaction.** The blood of most patients shows agglutinins from the fifth or sixth day of the disease, the titre usually rising to 1/640 or higher and then gradually falling during recovery.

The test is performed in the usual way. Dilutions of serum up to 1 in 2,560 should be used *as there may be a prozone even up to 1/640*. *Brucella* suspensions for the test may be obtained from the Standards Laboratory, Colindale, London, or prepared as phenolised heat killed suspensions of strains of known smoothness and standardised by opacity to match 1,000 million *Bact. coli* per cc. Tubes are incubated in a 52° C. water bath for 4 hours or over-night. Low-titre agglutinins may be readily missed unless the tubes are viewed with a hand lens against a dark background and compared before and after inversion with the control suspensions.

The agglutination reaction gives no information about the infecting type of *Brucella*. Frequently, however, an indication of the type may be obtained by differential absorption of the patient's serum. The principles involved are identical with those already discussed in describing the preparation of monospecific sera.

Interpretation of the results of the agglutination reaction is complicated by the fact that prolonged contact with infected animals or consumption of infected raw milk may give appreciable agglutinins in the absence of active infection. Moreover, as already stated, agglutinins may be absent even though blood culture is positive. Topley and Wilson suggest as a general rule that if there are no clinical symptoms a titre of 1/80 may be taken as an

indication of latent or past infection, a higher titre than this suggesting frequent attacks in exposed subjects like veterinarians; and that if there is pyrexia a titre of $1/80$ or over is suggestive of undulant fever, but a higher titre would be expected in an acute exacerbation of the disease in persons frequently exposed to it.

Whey Agglutination

Serological examination of milk is a useful and simple preliminary step in the detection of *Brucella*-infected herds. A variable proportion of cows infected with *Brucella* develop udder lesions associated with the excretion of *Brucella* in the milk and usually with the presence of *Brucella* agglutinins. The detection of agglutinins in milk is very easy if a clear whey is first obtained as follows:

Take 10 cc of milk, add 5 cc of chloroform to extract the fat, and a few drops of rennet. Incubate for an hour at 37°C . in a screw-top bottle and then centrifuge. The chloroform extract falls to the bottom owing to its high specific gravity, the intermediate layer contains the precipitated casein and on top is a clear supernatant whey. This latter is pipetted off and tested for agglutinins in the usual way.

A whey titre of 1 in 80 or over is very suggestive of udder infection; in heavily infected herds titres of up to 1 in 640 have been found even in bulked samples.

B. MOORE.

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CHAPTER IX

LABORATORY DIAGNOSIS OF ANAEROBIC INFECTIONS

INTRODUCTION

IN the past, a number of important infections associated with anaerobes has been largely neglected by the clinical pathologist, partly because of the technical difficulty of anaerobic culture and partly because the anaerobes, especially the non-sporing types, are for various reasons difficult to isolate on the simpler routine media. As an example of this comparative neglect it may be noted that the predominance of the anaerobic non-sporing bacilli in over 90 per cent. of adult human faeces was not recognised until it was demonstrated by Sanborn (1931), Torrey and Montu (1931) and Eggerth and Gagnon (1933), although cultivation of faeces had been practised for over fifty years. Another possible reason for this neglect is the small number of well-defined clinical syndromes with which anaerobes are constantly associated, compared with their relatively frequent occurrence in mixed culture in a number of ill-defined infective states.

Many of these obstacles disappear with the practice of routine anaerobic culture and it is the aim of this article to describe (a) anaerobic techniques simple enough to be practised as a routine and (b) easily prepared media that support the growth of even the more fastidious anaerobes.

Anaerobes are found in diverse situations both normally and in association with disease in man and animals. The *anaerobic streptococci* are normal inhabitants of the oral cavity, the intestinal tract and vagina and they have been found in purulent gangrenous disease of the genital tract, lungs and viscera, in septicaemia, in meningitis, and in both civil and military wounds. *Fusiform bacilli* are found in the normal upper respiratory tract and intestine; and in ulcerative processes of the mucous membranes of the throat, colon and vagina, in abscesses of the lung, liver, intestines, skin and subcutaneous tissues, and the breast, in osteomyelitis and gangrenous stomatitis (noma), in periurethral and other infections of the urinary tract and in septicaemia.

As an example of the importance of these organisms in clinical pathology we may note that non-sporing anaerobes (*fusiform*

bacilli and cocci) were found in about 4 per cent. of some 5,000 pathological specimens including pus, fluids, tissues, gall-bladder walls, bile and blood examined by Holicky (reported by Dack, 1940) and in 3 per cent. of blood cultures examined by Delbove and Reynes (1941). *Anaerobic actinomyces* are found normally in the mouth and throat, in lesions of muscle and bone and in empyemata: and *Clostridia* occur in puerperal infections, gut abscesses, endocarditis and in civilian and military wound infections.

Anaerobic gram-positive bacilli, chiefly Lactobacilli, occur in large numbers in faeces. In the adult they are not so common as the fusiform bacilli though they predominate in the stools of breast-fed children. Like other organisms in the faeces their presence is affected by diet (Eggerth and Gagnon, 1933). This group of organisms has not been specifically associated with any infective condition.

THE BACTERIOLOGY OF THE ANAEROBES

1. Fusiformis

The diversity of gram-negative, non-sporing organisms, ranging from cocco-bacilli to branching filaments, with an equally wide range of cultural and biochemical activities, is so great that the group almost certainly includes more than one genus. Weiss and Rettger (1937) distinguished two genera within the group, *Bacteroides* and *Fusobacterium*; the general name *Fusiformis* in this article is applied to all strictly anaerobic, gram-negative, non-sporing bacilli.

Fusiformis includes many morphological types from short bacilli to filaments, and although spiral forms occur it is generally agreed that they bear no generic relationship to the spirochaetes with which they are often associated in lesions (Hine and Berry, 1937; Spaulding and Rettger, 1937b). The morphology of fusiform bacilli changes considerably in subculture.

Subdivision of the group has been attempted on the basis of both morphology (Hine and Berry, 1937) and biochemical activity. Slanetz and Rettger (1933) suggested four subgroups; later Spaulding and Rettger (1937a) redistributed the strains on biochemical grounds disregarding morphological differences and arrived at a different four subgroups, with a few intermediate strains. The approximate relationship of the morphological to the biochemical subgroups is shown in Table I.

The species belonging to Group I of Spaulding and Rettger produce a foul odour in culture and their colonies on blood agar

develop a green zone on exposure to air; the species belonging to Group II produce no foul odour and remain non-haemolytic on exposure to air. Both groups are non-gelatinolytic. Of 11 strains of *Fusiformis* isolated by Henthorne, Thompson and Beaver (1936) from human inflammatory lesions, eight of which were fatal, five corresponded with Group II B and six with Group I A. *Fusiformis necrophorus* is in Group I A.

Bøe (1941) made a careful examination of the microscopic and colonial morphology, biochemical characters, serology and pathogenicity of 30 strains of *Fusiformis* isolated from pathological conditions of the respiratory system, from normal mouths and from a brain abscess. He concluded that differences between the strains were small and inconstant, and assigned them all to one species, *F. plauti-vincenti*. By reading his carbohydrate fermentation tests potentiometrically Bøe showed that all his strains were weak fermenters, the terminal pH never falling below 6.0. Results with the usual fermentation indicators were therefore unreliable. This observation opens to question the classifications based on biochemical reactions.

2. Anaerobic streptococci

The anaerobic streptococci may be divided into those that become aerobic on subculture and those that are strictly anaerobic. Further subdivision was made on the basis of colonial morphology by Colebrook and Hare (1933) and on the basis of microscopic morphology and biochemical activity by Prévot (1925, 1940). Stone (1940) examined 22 strains and found that the subdivisions based on the two classifications do not correspond and that there is no consistent correlation between colonial morphology, biochemical and serological properties. She considers that growth on 10 and 40 per cent. ox bile blood agar is sufficiently consistent to be a valuable differential character among the strictly anaerobic forms. Prévot's (1925) classification is given in Table II. It should be noted that many anaerobic streptococci found in pathological material fall outside the range of strains on which this table was based.

3. Actinomyces

Rosebury (1944) distinguishes two groups of Actinomyces, the parasitic forms whose natural habitat is the mouth and throat of man and animals, and the saprophytic forms whose natural habitat is the soil. Both parasitic and saprophytic Actinomyces may be pathogenic, but the saprophytic forms have been recovered only

rarely from actinomycosis in cattle and man. The two groups are compared in Table III (after Rosebury). There is as yet no basis for subdividing the parasitic group into separate species, and, though the group is probably heterogeneous, all members may provisionally be regarded as examples of the type species, *Actinomyces bovis*. For a fuller description and discussion of the pathogenicity of the anaerobic actinomycetes, see Erikson (1940).

4. Clostridia

The Clostridia are classified on morphological, colonial, biochemical and toxological grounds. The following tests are those most commonly employed for the identification of Clostridia.

Microscopic morphology gives only a broad basis of distinction between various Clostridia. In a given smear great variation is often found both in the morphology of vegetative forms and in shape and position of spores. It is important to assign to a morphological type only bacilli with mature, refractile spores and to assess the modal form from the examination of as large a number of organisms as possible.

Colonial morphology, on the surface and in the depth of agar, like microscopic morphology, gives only a broad basis of distinction. The appearance of the surface colony of a strain on ordinary strength agar may vary greatly depending on the moistness of the surface of the plate. For instance, *Cl. sporogenes* may appear on a dry plate as a tiny rhizoidal colony or on a moister plate as a flat smooth, fimbriate colony, or as any intermediate form. As the concentration of the agar is increased the colonies of the Clostridia often change in type.

Biochemical reactions, the chief of which for the better-established species are given in Table IV, are read after five days for carbohydrate fermentation and indole production; after seven days for milk and cooked meat broth reactions; and after three weeks for liquefaction of gelatine and coagulated serum. Indicators of acid production are liable to irreversible decolorization by growing anaerobes, the acidity of fermentation tubes should be judged by removing a loopful of culture and testing on a white tile against a drop of indicator, brom-thymol-blue being suitable for the purpose. Acids formed by fermentation are later neutralized by some actively proteolytic anaerobes; readings should therefore be made after one, three and seven days' incubation in order to detect both early and late acidity. Many clostridia form gas from peptone water; gas production is not of differential significance and Durham tubes

may be discarded if desired. The reactions shown by iron milk (see "Fluid media for anaerobic growth in air" below) may differ from those shown by plain milk incubated anaerobically and, in general, occur more rapidly and are greater in degree. On Loeffler's coagulated serum two degrees of proteolytic activity may be distinguished, disintegration and liquefaction. In both cases the medium breaks up when shaken, but in the former it does not liquefy even after prolonged incubation (one month). Variation in the brand of peptone may affect indole production (Reed, 1942); peptone used for this purpose should be tested against a number of known species of *Clostridia*.

Pathogenicity is usually tested in the guinea-pig and the mouse, using normal animals and control animals treated with specific antitoxin. A young culture in a liquid medium is injected intramuscularly, 0.8 cc. for a guinea-pig and 0.4 cc. for a mouse mixed with 0.2 cc. and 0.1 cc. sterile 12.5 per cent calcium chloride respectively. The calcium chloride produces in the tissues a necrosis favourable to the establishment of anaerobes, but it is also toxic to bacteria, so that the mixture should be inoculated immediately after preparation.

Death in the normal animal and protection of the control by a given antitoxin is taken to indicate that the unprotected animal has been killed by the exotoxin corresponding to the known antitoxin used. Thus, if a mouse is protected by an antitoxin of *Cl. welchii* against killing by a *Clostridium* that produces a fatal infection in the unprotected mouse, it is assumed that the *Clostridium* is *Cl. welchii*. Commercial therapeutic antitoxic sera prepared in the horse are commonly employed for this purpose. However, an antiserum of this kind may contain heterologous antibodies, either naturally, or as a result of previous immunizations, and its use for a diagnostic test is valid only if it has been titrated for heterologous antibodies and is used at a dilution at which they are ineffective. Even with this precaution the test may give an incorrect result. In the first place the antitoxin content may be insufficient to protect against an exceptionally virulent homologous strain; and in the second the antiserum may contain natural antibodies to an unknown or unrecognized heterologous toxin that happens to be produced by the *Clostridium* under test. If possible, diagnostic antisera should be specially prepared, immunization being restricted to only one antigen, injected into an animal whose history minimizes the chance of natural heterologous antibody production (for example, a laboratory-bred rabbit).

5. Lactobacilli

Originally all non-sporing, anaerobic bacilli from faeces were included in the *Bacteroides* (Castellani and Chalmers, 1919) but the gram-positive forms are quite unlike the gram-negative and have been transferred to the *Lactobacilli*.

Some anaerobic *Lactobacilli* become aerobic on subculture. These resemble Eggerth's *Lactobacillus bifidus* Type I. After a detailed study of the strictly anaerobic *Lactobacilli*, King and Rettger (1942) were unable to devise a satisfactory classification. They recommend that this group be called *Lactobacillus bifidus* Type II. It includes the eleven "species" of Eggerth (1935) and the three types of Lewis and Rettger (1940). *Lactobacillus bifidus* Type II is non-motile; is widely pleomorphic especially when rapidly sub-cultured; forms small entire-edged, slightly raised, granular colonies that can be moved whole on blood agar; ferments glucose, usually galactose, mannose and lactose, sometimes xylose, arabinose, rhamnose or more complicated glucosides like amygdalin, and never ferments dulcitol, sorbitol, inositol and the sodium salts of the carboxylic acids; seldom liquefies gelatine; seldom forms indole; and never produces H_2S .

GENERAL ANAEROBIC TECHNIQUE

The McIntosh and Fildes' anaerobic jar with Wright's capsule

The common procedure of making a primary culture in cooked meat medium when anaerobes are suspected may yield very misleading results. It is only from a knowledge of the relative numbers of viable bacteria of each species present in the lesion and their relation to the morphological varieties seen in direct smear that any conclusions about the causative rôle of the various bacteria can be firmly established. Direct plating is essential because culture in cooked meat medium, which favours the quickly growing species at the expense of the slowly growing species, yields only qualitative information about the flora of the lesion. For surface cultures on solid media the provision of an oxygen-free atmosphere is essential. Of the many modifications of the McIntosh and Fildes' anaerobic jar, that equipped with Wright's capsule (see Hayward, 1945) is simple, quick and easy to manipulate, and ensures good surface cultures of anaerobes. Anaerobiosis is produced by the combination of all the oxygen with excess hydrogen to form water, the reaction being catalysed by finely divided palladium. Most of the

palladium catalysts in use require heating. In Wright's capsule the palladium is reactive at room temperature; no heating is necessary and as a result the preparation of the jar is complete a few minutes after it has been filled with an adequate volume of hydrogen (see also Weiss and Spaulding, 1936).

The addition of carbon dioxide to the anaerobic atmosphere improves the growth of many strains of *Fusiformis*, *Lactobacilli* and *Actinomyces* so markedly that it should be made a routine practice. Two per cent. is sufficient. Up to 10 per cent. has been recommended, but the growth of many species is inhibited by this concentration. Carbon dioxide should be added to the jar after the evacuation of air and before the admission of hydrogen (see "Method of Use" below).

The Jar. Any airtight container with one tap is suitable, but metal jars are preferable because explosions occur occasionally. Plasticine is a good sealing agent, or a mixture of three parts of vaseline with one part of solid paraffin (M.P. 52° C.) may be used if the lid touches the jar over a fairly wide area.

The Capsule. One gram of palladium chloride is dissolved in 10 cc. of distilled water containing a few drops of concentrated hydrochloric acid; 1.5 g. of asbestos wool are soaked thoroughly in the solution, and dried in the incubator. The dry asbestos is teased out and heated in a smoky flame until black, and the black deposit then burnt off in a blow-pipe flame leaving a finely divided deposit of palladium on the asbestos fibres. The catalytic activity of this preparation may be tested by directing a fine jet of hydrogen on to it; within a few seconds it should warm up, glow, and ignite the hydrogen. This amount of catalyst is sufficient for six capsules. Each capsule is made by spreading the palladiumized asbestos loosely in a thin layer 34 mm. square on one half of a sheet of 30-40 mesh per 25 mm. copper gauze, folding over the unoccupied half of the gauze and closing the capsule by turning in the projecting 3 mm. edge of gauze on the three open sides.

These capsules are inactivated either by deposits of moisture on the fibres or by the reaction of the palladium with sulphur or arsenic. Damp capsules may be reactivated by drying in the outer cone of a Bunsen flame, which should be done each time the jar is set up, the capsule being allowed to cool while the jar is being filled with cultures. Sulphur inactivation may occur by the decomposition of hydrogen sulphide evolved by cultures in the jar. This inactivation can be reversed only by opening the capsule and re-roasting the palladiumized asbestos. A number of spare capsules

should be kept so that if one is found to be inactive it can be quickly replaced. The outer cone of the flame is recommended for the heating to drive off moisture. The inner blue cone contains unburnt gases including sulphides, which may poison the capsule. (For inactivation by arsenic, see next paragraph.)

The Hydrogen. If the H_2 is generated by the action of hydrochloric acid on zinc, it should be passed first through a 10 per cent. solution of lead acetate to remove H_2S and then a 10 per cent. solution of silver nitrate to remove AsH_3 . Electrolytically prepared H_2 need not be washed. If cylinders of H_2 are used, a reducing valve is necessary unless the H_2 is passed into balloons or football bladders from the cylinder and then led from the balloon to the jar through a water wash-bottle which acts as an indicator of hydrogen flow.

Method of Use. The jar is sealed and about $\frac{2}{3}$ of an atmosphere of air is removed and replaced by H_2 . After the first inrush of H_2 to replace the evacuated air, there is a pause, when H_2 ceases to bubble through the indicator bottle. Then, under the catalytic action of the capsule, H_2 again bubbles to replace the gases removed as water vapour. If this second bubbling is absent, the capsule is inactive, and should be replaced by an active one. The volume of H_2 admitted is more than sufficient to combine with all the O_2 present in the jar, so that the jar may be disconnected and transferred to the incubator as soon as the second inflow begins, since the capsule is designed to continue acting at incubator temperature.

Although this type of capsule is highly reactive, explosions are rare. It is probable that the explosive mixture of H_2 and O_2 , in which the ratio is as two to one, is detonated only when the capsule is at or above a certain temperature. If the jar is evacuated the full $\frac{2}{3}$ of an atmosphere and the H_2 is allowed to enter as quickly as possible the explosive proportions are present only while the capsule is still cold. The copper gauze should enclose the palladiumized asbestos completely to guard, on the Davy lamp principle, against explosions.

Indicator. Two per cent. glucose broth adjusted to pH 8.0 and coloured green with methylene blue will become colourless if it is in an anaerobic atmosphere. If a crystal of thymol is added it keeps indefinitely.

Concentrated Agar


On ordinary agar the colonies of most Clostridia are large and spreading, but their size may be minimized by over-drying plates

left open in the incubator for two hours, and separate colonies emerged by plating fresh dilutions of material or culture. However, the spreading of the aerobes *Proteus vulgaris* and *Ps. pyrogena*, and of the anaerobes *Cl. septicum* and *Cl. tetani*, cannot be controlled by drying alone. Various chemical inhibitors of spreading have been recommended, but most of them are inhibitors of growth. Spreading may be entirely inhibited by solid media containing high concentrations of agar.

The concentration of agar that inhibits spreading depends on the type of agar used and varies from about 4.5 to 7 per cent. It is determined by preparing a series of plates containing varying concentrations and staining them with *Proteus vulgaris*. As the concentration rises the colonies of *Proteus* become smaller and the lowest concentration that yields low convex colonies with an entire edge should be used. The addition of blood to agar media encourages spreading quite apart from its action as a diluent of the agar; the inhibiting concentration is therefore higher for blood agar than for nutrient agar, and must be determined in the presence of blood. Concentrated agar plates need drying only long enough to drive off the water of condensation on the surface of the medium.

The preparation of concentrated agar is made difficult by its high viscosity. Powdered agar is readily soluble but prolonged steaming is necessary to dissolve it. The resulting gel cannot be cleared by filtration, but most of the insoluble matter settles if the molten agar is allowed to stand in the steamer and the relatively clear supernatant layer is suitable for use. Enriching substances like blood must be mixed rapidly and thoroughly in the viscous molten agar, and plates poured quickly before the agar re-solidifies. The melting and solidifying temperatures of 4-8 per cent. agar gels are the same as those of 2 per cent.

Concentrated agar does not inhibit the growth of any species so far tested, including fusiformis, clostridia, staphylococci and streptococci both aerobic and anaerobic. It has the disadvantage of modifying colonial morphology, particularly of the clostridia. Other species vary less, but on concentrated agar they are on the whole smaller, rougher in texture, and sometimes crenated instead of entire.

The two disadvantages of concentrated agar, unfamiliarity of colonial appearance and difficulty of preparing media, are fully compensated for by the overwhelming advantage of having small discrete colonies of  permitting the ready isolation

of pure cultures from the primary plates even in the presence of spreaders like *Proteus vulgaris*.

Media

For clostridia, actinomyces and anaerobic cocci, enrichment of nutrient agar with blood or serum ensures growth, but the growth of fusiformis and lactobacilli in primary culture is greatly improved by additional enrichment with vegetable extract (potato, carrot, turnip, yeast, tomato or horse-radish). From a study of the growth requirements of the intestinal fusiform bacilli and lactobacilli, Lewis, Bedell and Rettger (1940) recommend a basal glucose-cysteine medium to which beef infusion and vegetable extract may be added

Glucose-cysteine Medium :

Tryptone (Bacto) 20 g, beef extract (Bacto) 10 g, glucose 5-10 g, cysteine hydrochloride 0.5 g, disodium phosphate (hydrate) 4 g, and either distilled water 1,000 cc or distilled water 400 cc, beef infusion 500 cc, and vegetable extract 100 cc

Vegetable Extract :

100 g raw vegetable (potato, carrot, turnip or horse-radish) ground by passing through meat grinder; infused with 100 cc. water at 8° C. for 24 hours; filtered through several thicknesses of cheese-cloth, then filter paper with suction pump, clear filtrate is boiled 2-3 min or until completely coagulated, refiltered through filter paper.

Fluid Media for Anaerobic Growth in Air

There is little need for growing fluid cultures in the anaerobic jar. Considerable jar space can be saved by the use of liquid media containing a reducing agent which supports the growth of anaerobes in the presence of air. Apart from the classical cooked meat granules two very convenient reducing agents are iron (Hayward and Miles, 1943) and sodium thioglycollate (Brewer, 1940).

Iron Media :

The addition of sterile 3 × 25 mm. strips of mild steel sheeting ("sheet-iron" about gauge No. 26) to ordinary laboratory liquid media allows the growth in air of even the strictest anaerobes from very small inocula. Except in the earliest stages, growth cannot be judged by turbidity because the medium becomes cloudy with iron hydroxides, especially if a reducing sugar is not present. However, the black precipitate which forms during the growth of the majority of anaerobes

may be taken as an indication of growth except in fermented carbohydrate media where the acid, which then indicates growth, prevents the formation of the black precipitate. It is advisable to store the iron separately and add it to media immediately before use.

Thioglycollate Media :

Brewer's medium is nutrient broth containing 0.1 per cent. of sodium thioglycollate (thiolacetate) and 0.05 per cent. of agar. The sodium thioglycollate may be prepared from the stable 90 per cent solution of thioglycollic acid obtainable commercially by exact neutralization to pH 7.2 with sodium hydroxide. About 11 cc of normal sodium hydroxide are required for 0.9 cc of the acid solution. This mixture, which must be freshly prepared for each batch of medium, contains about one gram of the sodium salt and is sufficient for one litre of medium. The medium must be tubed to a depth of at least 7 cm. and autoclaved, when it keeps for at least a month at room temperature. Broth containing 0.5 per cent. of agar and 1 per cent. of sodium thioglycollate is a useful concentrate that can be kept indefinitely in screw-capped bottles. If one part of concentrate is added to nine parts of any liquid medium it will support the growth of anaerobes in air.

Although liquid media may allow anaerobes to grow in air, they are anaerobic only in the depths. If it is desired that the growth of aerobes present in the inoculum should be as far as possible suppressed, these media should be incubated anaerobically.

Determination of Anaerobiosis

Agar shake cultures incubated in air indicate the atmospheric requirements of the strain. Strict anaerobes grow only in the depth, microaerophilic organisms grow throughout the medium but more profusely 1 to 2 cm. below the surface, facultative anaerobes grow throughout the medium but often more profusely in the surface layers, and strict aerobes grow only in the surface layers.

The Lethal Action of Air

Aerobic conditions are lethal to some anaerobes, notably *F. necrophorus* and *Cl. oedematiens*, and colonies may cease to yield successful subcultures within an hour of exposure to air. For this reason, at least 48 hours' incubation before examination is recommended for the initial anaerobic plate, and any later plates on which oxygen-sensitive organisms may be present; in addition, colonies should be picked into a liquid medium immediately plates are

removed from the anaerobic atmosphere. If a reducing agent, benzdine or sodium thioglycollate, is present in the medium, colonies remain viable in air for longer periods.

Time of Incubation

Up to ten days' incubation is necessary for the full development of colonies of the fusiform bacilli and actinomyces. If after 48 hours' incubation, which is usually sufficient for lactobacilli, anaerobic streptococci and clostridia, minute colonies are present or if direct smears show that slowly-growing species should be expected, incubation should be prolonged.

Special Notes on the Clostridia

Collection of Specimens from Infected Lesions. A sterile throat swab may be moistened with exudate from the depth of the wound but larger specimens are desirable; the most suitable are muscle or other tissue from the depths of the wound at the junction of healthy with necrotic and wound exudate collected with a short, wide-mouthed Pasteur pipette sterilized inside a test-tube. Muscle specimens should be extracted with broth or peptone water.

Microscopic examination. Examination of a film should never be omitted. Occasionally it may be the only means by which an early clostridial myositis can be certainly differentiated from streptococcal myositis. The films should be gram-stained; it should be remembered that many clostridia are easily decolorized and may appear gram-negative; spores are readily visible in gram-stained preparations. *Cl. welchii* is rarely seen in the sporing stage.

The film may show (a) a large variety, and possibly large numbers of organisms; in this case there is probably no serious infection with clostridia; (b) a preponderance of bacilli presenting a fairly uniform picture; this is very suggestive of clostridial infection; pus cells are scanty and degenerate in serious cases of gas-gangrene. (c) enormous numbers of streptococci, some of the small type characteristic of certain anaerobic streptococci; this is typical of streptococcal myositis; pus cells are then numerous. If either non-pathogenic clostridia or aerobic spore-bearers are present in streptococcal myositis, or if streptococci are present in clostridial myositis, the number of pus cells may be the only feature distinguishing streptococcal from clostridial myositis.

The Nagler Reaction for *Cl. welchii* and *Cl. bifermentans*. The Nagler reaction depends on the decomposition of soluble lipoprotein complexes in human serum by certain clostridia and its specific inhibition by the alpha-antitoxin of *Cl. welchii*. It may be used for the rapid identification of *Cl. welchii* and *Cl. bifermentans*. It is demonstrable in liquid or on agar media containing human serum (Hayward, 1941 and 1943). In liquid media it appears as an opacity or a floating curd of lipoid material; on agar media as a conspicuous opacity in the agar surrounding the colonies of reacting clostridia. Since a number of spore-bearing bacilli, aerobic and anaerobic, and certain other species of bacteria also produce opacity in human serum media, a medium containing the alpha-antitoxin of *Cl. welchii* must always be tested in parallel. The Nagler test is positive only if there is complete inhibition by the antitoxin in the control medium.

The Nagler Plate

Add 20 per cent human serum and 5 per cent. Fildes' extract (peptic digest of sheep's blood) to molten nutrient concentrated-agar at 50° C. Pour the plate, dry off water of condensation, and mark into halves. On one half spread 2-3 drops (about 0.1 cc., 50-100 international units) of *Cl. welchii* antitoxin and allow to dry. Inoculate both halves of the plate with the swab, spreading the inoculum in the same pattern on each side.

The human serum may be added to any medium which encourages both growth and toxin-formation by *Cl. welchii*, provided that it is clear enough to permit the detection of the Nagler opacity. If desired, a separate control plate containing about 4 units of antitoxin to the cc. may be used. A batch of human serum which gives a good reaction should be selected, it will keep its full Nagler-reacting properties for several months at 2° C.

The Nagler Tube Reaction

If the Nagler reaction of a pure culture is to be tested a tube reaction is more economical than the plate test described above. For this, two tubes containing equal parts (e.g. 0.15 cc.) of human serum and broth containing 5 per cent. of Fildes' extract are inoculated with the culture under test, and to one a drop of *Cl. welchii* antitoxin is added. When growth has occurred the tubes are centrifuged and read for opacity.

Spore Formation and Differential Heating. Resistance to heating at 80° C. for 10 minutes is usually taken to indicate the presence of spores, though it must be remembered that certain non-sporing organisms will survive heating of this degree.

Differential heating may be used for the separation either of sporing organisms from vegetative forms (80° C., 10 minutes) or of the more heat-resistant sporing forms (e g. 100° C., 5-20 minutes). Heating at 100° C. may be used for the isolation of *Cl. oedematiens*, a species that grows poorly but whose spores are relatively resistant to heat.

DISCUSSION

The occurrence of the anaerobes as part of the normal flora of the mucous membranes, the very mixed flora of lesions with which anaerobes are associated, and the negative or at best indefinitely positive results of pathogenicity tests on pure cultures of non-sporing anaerobes isolated from the lesions make it very difficult to assess the rôle of these bacteria in infection. It seems probable, that they often act synergistically. Thus a mixture of aerobic and anaerobic bacteria isolated from periodontal abscesses in man will cause the rapid death of guinea-pigs inoculated subcutaneously, whereas each organism alone, or the mixture lacking any one of its component species, is non-pathogenic. There is evidence too that certain mouth and lung lesions are not caused by single species but by a combination; results in experimental animals suggest that the clostridia are enhanced in virulence by association with other bacteria, including aerobes.

Another factor is the predisposing influence of the lowered resistance of tissues owing to a metabolic disturbance like an avitaminosis. There is evidence, both clinical and from animal experiments, that although the non-sporing anaerobes are secondary invaders of already diseased tissues they are probably responsible for the suppuration which is so characteristic of the lesions with which they are associated.

Until the significance of the various anaerobes in infection is made clear from a retrospective analysis of case histories and from assiduous surveys of the flora in all kinds of infections, no useful generalization will be forthcoming. It is to these ends that it would seem best to direct future investigation.

TABLE II
ANAEROBIC STREPTOCOCCI

Strict anaerobes Lenticular colonies in deep agar				Facultative aerobes	
Gas and foetid odour Milk not coagulated Gelatine not liquefied		No gas, no foetid odour		Irregular colonies in deep agar	
Diameter 0.8-1 μ In neutral broth, short chains showing bending in a di- rection which is either oblique or per- pendicu- lar to the prin- cipal direction, granular sediment no tur- bidity	Diameter 0.8 μ In neutral broth, chains long and regular		Diameter 0.3 μ Milk not coagula- ted Ge- latine not liquefied	Diameter 0.6-0.7 μ Milk co- agulated. Gelatine not lique- fied.	Milk co- agulated No gas; no foetid odour Gelatine liquefied Diameter 0.7 μ
	Gas in or- dinary media Granular sediment, no tur- bidity	No gas in ordinary media, gas in media contain- ing fresh tissue Uniform turbidity			
<i>M. foetidus</i>	<i>Str anae- robis</i>	<i>Str putridus</i>	<i>Str micros</i>	<i>Str inter- medius</i>	<i>Str evolutus</i>

TABLE III
COMPARISON OF THE PARASITIC AND SAPROPHYTIC
ACTINOMYCETES

	Parasitic Actinomycetes	Saprophytic Actinomycetes
Natural habitat	Mouth and throat of man and probably of cattle and other animals, obligate parasites; sometimes pathogenic	Soil, grains and grasses, widely distributed in nature; some pathogenic species, but most forms are non-pathogenic
Cellular morphology	Branched mycelium, gram-positive, not acid-fast. Marked tendency to fragment into bacillary forms	Branched mycelium, gram-positive; some are acid-fast. Generally little tendency to fragment into bacillary forms
Character of growth	Bacteria-like colonies without aerial hyphae; no spores; no pigments	Colonies more mould-like, often with aerial hyphae and spores (conidia); many produce yellow, orange or black pigments
Temperature requirements	Optimum, 37° C; no growth at 22° C.	Optimum usually 15-20° C.
Relation to oxygen	Oxygen tolerance limited; generally fail to grow or grow poorly under aerobic conditions	Aerobic, some forms do not grow anaerobically.
Metabolism	Probably never proteolytic. Ferment carbohydrates with production of acid.	Many forms actively proteolytic; may utilize carbohydrates without acid production
Pathogenicity	Causative agent of true actinomycosis in man and animals	Occasional causes of an actinomycosis-like disease, very rare in man, and of tropical cutaneous mycetomas, e.g. Madura disease

TABLE IV. CLOSTRIDIA

	Pathogenicity to laboratory animals	Cooked meat	Spores	Iron nitrate milk	Liquefaction of gelatin	Digestion of serum	Glucose	Maltose	Mannitol	Lactose	Sucrose	Salicin	Glycerol	Remarks
<i>welchii</i>	+/-	G	OS	A, C/- G/g	+	-	AG	AG	-	AG	AG	-/AG	AG/-	Sometimes cap- sulated. Non- motile
<i>oedematis</i>	+/-	G	OS	a, C/+ g	+	-	AG	AG	-	-	-	-	AG/-	.
<i>septicum</i>	+	G	OS	A, C/+ g	+	-	AG	AG	-	AG	-	AG	-	
<i>fallax</i>	+/-	G	OS	A, C/- g	-	-	AG	AG	AG	AG	AG	AG	-	
<i>haemolyticum</i>	+	G	OS	A, C	+	-	AG	-	-	-	-	-	AG	
<i>difficile</i>	+	G	OS	g	-	-	AG	-	AG	-	-	AG	-	
<i>carnis</i>	+	G	OS	G	-	-	AG	AG	AG	AG	AG	-	-	Feeble aerobic growth
<i>chauvoei</i>	+	G	OS	A, C g	+	-	AG	AG	-	AG	AG	-	-	
<i>tetani</i>	+/-	od/-, g/- d/-, B/-	RT	a, P/-	+/-	±	-	-	-	-	-	-	-	Forms indole
<i>histolyticum</i>	+/-	od, d XtIs	OS	a, g P → D	+	+	A/-	A/-	-	-	-	-	-	Feeble aerobic growth
<i>bifermentans</i>	+/-	OD, G d, B XtIs	OS	a, g P → D	+	+/-	AG	AG	-	-	-	-/AG	AG	Forms indole

Cl. botulinum	+	OD, G d, B	OS	P → D	+	+	+	AG	AG	-	AG/-	-	-/AG	AG	Sometimes cap- sulated
Cl. butyricum	-	G	OS	A, C G	-	+	+	AG	AG	?	AG	AG	AG	AG/-	
Cl. multifermentans	-	G	OS	A/a, C/- G/g	-	-	-	AG	AG	-	AG	AG	AG	AG	
Cl. cochlearium	-	-	OT	-	-	-	-	-	-	-	-	-	-	-	
Cl. tertium	-	G	OT	A, C/- g	-	-	-	AG	AG	AG	AG	AG	AG	-	Grows aerobically
Cl. paraputrificum	-	G	OT	A, C G/g	-	-	-	AG	AG	-	AG	AG	AG	-	
Cl. sphenoides	-	G	RT	A, G	-	-	-	AG	AG	AG	AG	-	AG	-	Sometimes forms indole
Cl. tetanomorphum	-	G	RT	a/-	-	-	-	AG	AG	-	-	-	-	-	Sometimes forms indole
Cl. hastiforme	-	-/od	OS	P → D	+	-	-	-	-	-	-	-	-	-	
Cl. capitovale	-	od, G B	OT	a, g P → D	+/+	-	-	AG	+	-	-	-	-	-	Sometimes forms indole
Cl. sporogenes	-	OD, G D, b	OS	a, g P → D	+	+	+	AG	AG	-	-	-	-/AG	AG/-	
Cl. aerosolactidum	-	OD, G D, b	OS	a P → D	-	-	-	AG	AG	-	AG	-	-/AG	-	
Cl. putrificum	-	OD, D b	RT	P → D	+	+	+	-	-	-	-	-	-	-	

+ = positive ± = partial - = negative. G or g = gas. Od or od = odour. D or d = digestion. B or b = blackening
 Xtls = late deposit of white crystals O = oval. R = round (spherical). S = subterminal or equatorial T = terminal. A or
 a = acid C = clot. P = precipitate. Small letters indicate a lesser degree of change. Reactions separated by a stroke like +/-
 indicate that the activity is not a characteristic of all members of the species; the common reaction is given first. P → D = precipitation
 followed by digestion.

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CHAPTER X

LABORATORY CONTROL OF PATHOGENIC STAPHYLOCOCCI

FOR many years the staphylococci were, as far as the clinical laboratory was concerned, relatively neglected. No finality had been reached concerning which of their easily recognised biological properties run parallel with potential pathogenicity. Minor variations in the technique of different workers made it difficult to compare and assess the clinical value of laboratory observations. But in recent times widespread interest in the problems of accidental infection in operating theatre and ward, the control of wound sepsis and the advent of the sulphònarnides and penicillin has provoked new and well-organized attempts to devise some system by which pathogenic strains can be recognized with reasonable speed and certainty. With the staphylococci the particular reactions to which for this purpose attention should be given are the production of coagulase, haemolysin and pigment, the fermentation of mannitol and lactose and the liquefaction of gelatin. Although the majority of highly toxigenic strains give positive results in all these respects it has gradually come to be accepted that the first, namely coagulase production, is the most significant, confirming the view of Cruickshank (1937) that only those strains which produce significant amounts of coagulase deserve the name of *Staphylococcus pyogenes*. However, in doubtful strains the other properties may still have some importance and therefore all will be mentioned, though briefly, in the review which follows.

The Coagulase Test

The reaction itself, first described by Much (1908), is easy to demonstrate but not so easy to control quantitatively. If an attempt is to be made to correlate the reaction quantitatively with possible pathogenicity, there are several technical fallacies to be avoided. Gillespie (1943) has reviewed these clearly and suggested standard methods which work well.

Sources of Error. The test must be made with human or rabbit plasma. Various common contaminants such as *B. subtilis*, *Ps. pyocyanea* and *Bact. coli* are known to clot some animal plasmas but most observers agree that they never thus affect human plasma which is clotted by the staphylococcus only. The culture medium

should be simple digest broth and each batch should be tested to see that it does not inhibit coagulation. The addition of glucose or other accessories to broth may cause marked inhibition. For the same reason human plasma should not be used if derived from blood to which glucose has been added. "Liquoid" is also an inhibitor, as may be chemical impurities in the saline. Finally, clotting may be prevented if the test is made upon cultures incubated in the presence of CO_2 in order to demonstrate haemolysin (Gillespie *et al.*, 1939; Dirocco and Fulton, 1939).

Technique of the Test. The method of Gillespie is given: "To 0.5 cc. of a 1 in 10 dilution of human plasma (oxalated or citrated) add from a capillary pipette five drops (approximately 0.1 cc) of an 18 to 24 hour broth culture. The result is read after 1 and 3 hours' incubation at 37°C . Thereafter the tubes are left at room temperature and read again next day. If pure cultures are used a coagulum should appear in $\frac{1}{2}$ to 1 hour, but occasionally it does not appear for 3 hours at 37°C . or for a further 18 hours at room temperature. Thus the test should never be regarded as negative until the tubes have stood at room temperature for 18 hours."

The Test in Practice. The time factor is of importance in a test upon the result of which may depend a variety of clinical or administrative procedures. In tests of specimens direct from the patient two cultures should be made (a) upon a blood agar plate and (b) in a good volume of broth. After overnight incubation the former will indicate the purity or otherwise of the growth, the latter will be available for the coagulase test. If the broth culture grows staphylococci only it may be used for the test: Gillespie has shown that satisfactory positive results are obtained when not all the staphylococci belong to the true *pyogenes* variety. If the mixed broth culture contains *Proteus* or *Streptococcus haemolyticus*, both of which may produce lytic substances, false negatives are liable to arise and the staphylococci on the blood-agar plate should then be used for the test after subculture in broth.

The Slide Agglutination Test. An indirect method of detecting coagulase-positive strains is by slide agglutination, as developed from the observation by Birch-Hirschfeld that a plasma which is coagulated by a particular staphylococcus will "agglutinate" the staphylococcus when mixed with it on a slide. Clumping is easily visible to the naked eye and takes place quickly, but most observers agree that the method gives a proportion of false positives and false negatives. The method of Cadness-Graves

et al. (1943) may be recommended "Place a clean slide and a clean, grease-free slide side by side, and put a drop of water on each with a 2 mm. loop. Rub the suspected colony into the first drop, with a minimum of spreading, and when the suspension is homogeneous, transfer the wet loop to the other drop (on the grease-free slide) and spread it into a thin film for staining. The amount of growth should be large enough, or the volume of the water should be small enough to produce in the first drop a watery suspension equivalent in opacity to at least 100×10^9 *Bact. coli* per cc. Mix one very small loopful of fresh human plasma into the first drop, keeping up a circular motion, 3-5 per second, for 10 seconds. If clumping occurs, a presumptive diagnosis of *Staph. pyogenes* may be made, confirmed by the appearance of the stained smear."

Fallacies associated with the fact that a very few staphylococci are auto-agglutinable in water alone are avoided by the above double-drop technique.

Fermentation of Mannitol

Pride of place as a test of potential pathogenicity belongs to the coagulase technique, but simple fermentation tests can be helpful; overnight fermentation of mannitol usually indicates pathogenicity and the procedure is useful as a simple presumptive test. Fermentation tubes with 1 per cent. mannitol and at pH 7.6-7.8 with phenol red as indicator are used. Inoculation is made with a single drop of a saline emulsion from a surface growth diluted to a standard opacity of approximately 2,000 million staphylococci per cc.

Haemolysin Production

It is so often assumed that description of a strain as being haemolytic implies its pathogenicity that a word of caution is necessary. It is generally agreed that the staphylococcal *alpha*-haemolysin, active at 37° C. against both rabbit and sheep erythrocytes as originally demonstrated by Glenny and Stevens is related to human pathogenicity. But there is a definite lack of correlation between test-tube detection of the haemolysin in the filtrate and its observation as a result of surface growth on a blood-agar plate. Hallman (1937) and McFarlan (1938) have both demonstrated the frequent occurrence of "haemolytic" strains on blood-agar which were coagulase negative, but in the filtrate many observers, *e.g.*

Gillespie, Devenish and Cowan (1939), have found relatively complete agreement between haemolysin and coagulase production. The following technique is satisfactory:

Subcultures in 0.5 cc. nutrient broth ($3 \times \frac{5}{16}$ in. tubes) are incubated at 37° C. for 48 hours in air containing 30 per cent. of carbon dioxide. The cultures are centrifuged and 0.1 cc. of the supernatant fluid tested for *alpha*-haemolysin against a 2 per cent. suspension of rabbit red corpuscles. A control tube containing one unit of staphylococcal *alpha*-antitoxin should be set up for each strain. The tubes are examined for haemolysis after an hour at 37° C. and 18 hours on the bench.

Detection of *alpha*-haemolysin production is an indication of potential pathogenicity and runs closely parallel to the coagulase test but in clinical work is perhaps best reserved as confirmatory.

Gelatin Liquefaction

The great majority of pathogenic strains liquefy gelatin and this test may be helpful when other reactions give equivocal results.

A single stab culture is made in a gelatin tube and incubated at 22° C. Liquefaction obvious within 72 hours is suggestive of pathogenicity; the probability decreases the longer it is delayed. Most non-pathogenic strains fail to liquefy the gelatin even after several days.

Grading: During a period of three years Knott and Blaikley (1944) attempted by regular swabbing of all the usual sites to exclude from a maternity department possible carriers of pathogenic staphylococci among the patients and the staff. On statistical evidence they seem to have had some degree of success since, after institution of this method of control, the effectiveness of the department, as judged by turnover of cases, increased by about 25 per cent. In order to detect strains likely to be invasive, all the above tests were applied, and the staphylococci were "graded" according to the results generally as A, B, C or D. All carriers of A strains were immediately excluded or isolated, and also the great majority of the B. In summary, A strains are positive for coagulase within 6 hours and B a few hours later. A strains ferment mannitol overnight; with B strains it is observed during the next day. Haemolysin and pigment production are observed upon a 5 per cent. rabbit blood-agar plate. With this method weak production of either does not exclude from being in Grade B, strong production tends to upgrade. Gelatin liquefaction was also tested and

recorded as mentioned above. Results supported the view that the coagulase and mannitol test are the most constantly related to pathogenicity.

IDENTIFICATION OF PARTICULAR STRAINS

Serum Typing

For accurate epidemiological work pathogenicity tests alone provide insufficient information. Some form of "typing" is essential. Progress in this direction was made by Cowan (1939) in his classification of staphylococci by slide agglutination. He first differentiated three main types and later distinguished several subtypes within them. The method has produced useful results but its epidemiological value is limited because of the small number of types.

Phage Typing

A much more promising method employs a series of staphylococcal bacteriophages according to the principle recently suggested by Fisk (1942). The number of different phages and the specificity is such that a technique with standard phages will probably soon be available. Wilson and Atkinson (1945) have recently described a method for obtaining purified and potent staphylococcal phage filtrates which has allowed them to identify 21 different staphylococcus types. Of 400 fully tested staphylococcus strains, 60.4 per cent. were typeable, 22 per cent. were sensitive to phage but could not be typed, and 17 per cent. were insensitive to their phage filtrates. They have demonstrated the usefulness of phage typing in tracing sources of infection in outbreaks of staphylococcal food poisoning, pemphigus, breast-abscess, etc. Their technique for the preparation of phage filtrates and for staphylococcus typing is described below.

Preparation of Crude Filtrates: Strains to be examined for latent phage contamination were grown in 0.2 per cent. glucose broth for 18 hours at 37° C. Each strain was then used in turn as a "basal" strain. Four drops (0.1 cc.) of the culture were spread over the whole surface of a 0.2 per cent. glucose-agar plate, and, after these had dried, the remaining cultures were "spotted" on to it. When the drops had been absorbed, the plates were incubated at 37° C. for 6-8 hours and left at room temperature overnight.

Phage action was revealed next morning by the presence of either discrete plaques or a narrow zone of inhibition of growth, less than 0.5 mm. in diameter, around the edge of the spotted culture. The growth of any pair of strains showing evidence of phage action was scraped off the agar and suspended in a small volume of broth. This was centrifuged, and the supernatant fluid was tested against both strains to find out which was the lysogenic and which was the susceptible strain, and the remainder of the lytic supernatant fluid was added to a 2-4 hour 0.2 per cent. glucose-broth culture of the susceptible strain and incubated at 37° C. for 4-6 hours. As soon as evidence of lysis became apparent, the culture was added to fresh culture of the susceptible strain and again incubated for 4-6 hours. Usually cultures were left overnight on the bench, but if it was not practicable to continue serial passage on the following day they were transferred to the ice-chest. The number of passages required to obtain a sufficiently high degree of lytic activity varied usually from four to twelve. The final culture was filtered through a Seitz EK pad and tested against the susceptible strain by the agar-plate method. The result was regarded as satisfactory if the pure filtrate produced confluent lysis.

Purification of Filtrates: The crude filtrates thus obtained were plated out with the susceptible strain, and a single plaque was picked off and spread on a 0.2 per cent. glucose-agar plate. This plate was incubated for 6-8 hours and then kept at room temperature overnight. A fresh plaque was picked next morning and the process repeated once. The growth from the second plating was scraped off, suspended in 5 cc. of glucose broth culture and centrifuged. The phage in the supernatant fluid was then carried through a series of passages in glucose-broth cultures, as described in the preparation of the original filtrates, until a potent lytic agent had been obtained. The final filtrate was kept in the ice-chest without preservative.

Titration of Lytic Filtrates: The Seitz filtrates from the last serial broth cultures were diluted 1/10 to 1/1,000,000 with quarter-strength Ringer solution. Four drops (0.1 cc.) of an 8-10 hour broth culture of the susceptible strain were spread evenly over the surface of a glucose-agar plate, allowed to dry, and then "spotted" with a standard loopful of each dilution of the filtrate. Plates were incubated in the usual way for 6 hours, kept at room temperature overnight, and read next morning. The highest dilution producing confluent lysis was regarded as the "test dilution"—i.e. that suitable for typing tests.

Preparation of Lytic Filtrates from Untypeable Strains: Strains that showed no phage action with the test dilutions of the available lytic filtrates were tested with the undiluted filtrates. If evidence of phage action was observed with any filtrate, a single plaque was picked off with some of the surrounding growth, and spread over a glucose-agar plate. Propagation of the phage was then carried out by the method already described.

Method of Setting up Tests: Nutrient agar plates containing 1.5 per cent. of agar and 0.2 per cent. of glucose are dried at 37° C. for an hour with the lid partly removed. The strain to be typed is grown in 0.2 per cent. glucose-broth for 8-10 hours, and four drops of the culture are spread evenly over the surface of a plate. After about 15 minutes, when these have dried, a standard loopful—or, if several strains are being tested, a drop of appropriate size from a Pasteur pipette—is "spotted" on to the culture. For the sake of convenience the bottom of the plate is divided by a glass-writing diamond into 25 half-inch squares, so that 25 lytic filtrates can be tested, if desired, on any one strain. When the drops have been absorbed, the plates are incubated for 6-8 hours, left at room temperature overnight and read next morning. The scheme of notation adopted is as follows:

Confluent lysis with no secondary growth	++++
Confluent lysis with secondary growth .	+++
Numerous semiconfluent plaques . . .	++
Discrete plaques	+
Less than 20 plaques	±

RESISTANCE TO STAPHYLOCOCCAL INFECTIONS

Phagocytic Power: The occurrence of clinical staphylococcal infection depends, not only upon the potential pathogenicity of the organism, but also upon the patient's resistance. Note may be made of the need, particularly in the younger age groups not only to test the urine for glucose, but if necessary, to perform a glucose tolerance test. In older people, especially if an eczematous element is present, renal function may be an important consideration. The blood count, quite apart from eliminating the possibility of any of the "primary" blood diseases, will show whether a full supply of polymorphs is present, and, when the infection is really active, whether there is a satisfactory shift to the left in the Arneeth count. A leucocytosis over 15,000 per cmm. is a common finding in deep-seated staphylococcal infection. Although the blood count may

show a satisfactory white cell response, the power of the polymorphs to ingest staphylococci, especially the infecting strain, may be poor. A simple way of observing this is to make a lightly cloudy suspension of staphylococci from an overnight agar culture in 2 per cent. citrate. In a clean, dry, 1 in 100 blood-counting pipette draw up this suspension to mark 1.0. Take this into the bulb. Clean and lightly prick the patient's finger as for a blood count, express blood and draw up seven fillings of the pipette to the mark 1.0. Mix the emulsion and the blood thoroughly by rotating the pipette during taking; at the end collect all the blood and citrate together in the bulb and mix with the aid of the bead. The process is repeated using another pipette and normal blood as a control. The rubbers of the two pipettes are slipped over the tips so that both are closed and drying prevented, and the pipettes incubated at 37° C. for twenty minutes. After removal again mix by rotating, express drops on to a clean slide; spread films and stain with Leishman. The phagocytic activity of patient's and control leucocytes are thus easily compared.

Staphylococcal Antibodies in the Serum : The staphylococcal toxic products proved to be antigenic are the haemotoxins and leucocidin. Coagulase is non-antigenic. The physical and antigenic properties of the *alpha*-haemolysin appear to correspond most closely to those of the "staphylococcus exotoxin" commonly prepared by growing staphylococci in broth media for a few days in an atmosphere of 20-40 per cent. CO₂, and from which the therapeutically used staphylococcal toxoid is made by exposing the filtrate to 0.3-0.5 per cent formaldehyde at 37° C. for 7-20 days. Estimation of the antihaemolysin developing in a patient's serum was at one time thought to give a reliable indication of response to immunisation. In the majority of patients an increase in antihaemolysin does not necessarily correspond with clinical improvement. Possibly active production of antileucocidin may more certainly indicate rising resistance. According to Valentine (1936) its production is of chief importance in the development of staphylococcal immunity and therefore toxoid for therapeutic use should be prepared from strains strong in both leucocidin and *alpha*-haemolysin production. The present custom is to use injections of both vaccine and toxoid to stimulate staphylococcal immunity.

SPECIFIC THERAPEUTIC PRODUCTS

Vaccines and Staphylococcal Toxoid : The literature is conflicting as to the therapeutic value of vaccines and staphylococcal

toxoid My experience is that after exclusion of primary conditions known to lower resistance to staphylococcal infection, results in mild chronic cutaneous lesions are generally favourable. One cause of failure is the undiscovered chronic focus of staphylococcal infection, such as antrum, small sinus or tooth socket. A combined course of vaccine, preferably autogenous, and toxoid gives better results than toxoid alone. The two may be given together in the same syringe but are better stored in separate bottles. The average initial dose of vaccine has been 30 million organisms with an increase of approximately 30 per cent at each successive dose up to a total adult dose of 1,500 millions. The initial dose of toxoid has been 0.1 cc; it tends to produce a painful reaction and should be increased cautiously; it may be well to start with a 1 in 10 dilution of the full strength. If improvement is to be brought about it is usually evident at the end of a course of about one dozen injections. It is seldom worth while persisting beyond this. On the other hand if good results are quickly apparent it is advisable to complete a course of about a dozen injections with a view to establishing firm immunity. In patients subject to persistent attacks of boils and furuncles, periodical courses of injections may be advisable. The interval between injections should never be less than three days. In commencing a course, injections may be given twice weekly but should not be repeated so long as there is any sign of reaction at the site of the previous injection. After the first two weeks, intervals of at least one week between doses are advisable.

Vaccine therapy is useless in acute staphylococcal infections. In these staphylococcal antitoxin may be of great value; even with the introduction of the sulphonamides the serum still retained its uses; many infections which would respond to neither separately proved susceptible to the two given together. The introduction of penicillin has completely changed the outlook in acute staphylococcal infections; whether serum will retain any value in combination with penicillin remains to be seen.

Appendix

Quantitative Blood Culture

Quantitative blood culture gives valuable information in the prognosis and treatment of staphylococcal septicaemia. McLellan and Goldbloom (1942) suggest that frequent blood cultures bring to light many unsuspected cases of such septicaemia. Diminution of the number of organisms

in the blood indicates localization and improvement of the outlook and also gives a guide as to the efficacy of the therapeutic measures employed. A simple method of quantitative blood culture is:—

Take to the bedside 3 to 6 tubes each containing 10 to 12 cc. melted agar, still standing in a small bath of warm water, together with a corresponding number of sterile Petri dishes. Withdraw blood by venepuncture using a sterile dry syringe; inject 1 cc. of blood into each of the agar tubes and pour immediately into the plates. After incubation colony counts can readily be made.

Counts of less than 20 organisms per cc. of blood usually indicate some localization of the infection, and a more favourable prognosis. An initial count over 20 organisms per cc., or a sharp increase in numbers on two successive days indicates a septicaemic condition which is likely to prove fatal unless effective treatment, e.g. with penicillin, is begun.

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CHAPTER XI

LABORATORY CONTROL OF CHEMOTHERAPY

THE term *chemotherapy*, originally coined to define treatment of parasitic infections with synthetic compounds such as dyestuffs and arsenicals, is now widely used to cover the treatment not only of parasitic, but also of bacterial and virus diseases, with a wide range of substances, the basic feature of which is that they are less toxic to the host's cell than to that of the infective agent. Their effectiveness in this respect is usually shown by the *therapeutic ratio*, a value conveniently expressed by the experimental formula :

$$\frac{\text{L.D. } 50 = \text{average lethal dose } ^1}{\text{C.D. } 50 = \text{average curative dose}}$$

but as might be expected of a group of substances embracing compounds so different as quinine, salvarsan, chaulmoogra oil, the sulphonamides and penicillin, their modes of action vary widely and are often obscure.

This chapter deals only with that section of chemotherapy which is related to laboratory work in clinical pathology, namely *anti-bacterial chemotherapeutics*. Three groups of substances exist which have antibacterial properties.

The first group are compounds which interfere with the normal bacterial metabolism in a highly specific way, e.g., the sulphonamides or the pantoyl taurine group. Their specific characteristic is that chemically they are closely related to growth factors required by bacteria, so that by blocking specific enzymes they stop the synthetic anabolic process in the bacterial cell.

The second group are metabolic products of bacteria or moulds. Often of unknown chemical composition, they inhibit certain bacteria, and are thus known as *antibiotic substances* (or mycoins). To this group belong penicillin, pyocyanase, gramicidin, clavacin; also enzymes such as lysozyme, hyaluronidase, protein extracts from wheat germ, and others, which are capable of destroying or modifying the bacterial cell by inducing chemical changes in its structure.

¹ The therapeutic ratio is the relation between the toxicity of a given substance and its efficiency as a curative agent. The "L.D. 50" is the median lethal dose; i.e. that which kills 50 per cent. of the experimental animals. The "C.D. 50" is the median curative dose; that is, the dose sufficient to cure 50 per cent. of the treated animals. Median values are now in general use, being preferred to minimal or maximal values.

The third group includes practically all those antibacterial substances known as antiseptics or disinfectants. Some of these act as gross protoplasmic poisons, destroying the general catabolic cellular mechanism by forming true chemical products (albuminates) with the bacterial tissue. Other antiseptic substances act as oxidising or reducing agents or by modifying the osmotic pressure. (For a general description of the properties and mode of action of antiseptics, see Garrod, 1940). Newer research on these groups of antiseptics has proved that many mercury, arsenic, or acridine compounds attack bacteria not by direct chemical action causing death, but by interference with a metabolite essential for the bacterial cell and therefore reversible. (See Fildes, 1940.)

In view of these findings, the clear distinction between a bacteriostatic and bactericidal action is often impossible to establish. Bacteria exposed to mercury perchloride solution are apparently dead, but if they are washed with sulphhydryl or cultivated in a thioglycollate medium even after some hours' contact with perchloride, growth occurs. Gentian violet is regarded as an antiseptic agent against gram-positive bacteria, but if the medium is sufficiently acidified to produce dissociation of the dye-bacteria complex the seemingly dead bacteria recover and grow. For the elimination of the infecting organism from vital tissues we demand not that the basic feature of a curative substance should be bactericidal action, but that it should be bacteriostatic and at the same time have suitable pharmacological properties—low toxicity combined with chemical stability in the body. The body-tissues, if sufficiently healthy and reactive, may be expected to "complete the kill" of the invading pathogen. Therefore the term "antiseptic" should be applied in a more biological sense, purely germicidal action being attributed only to "disinfectants" which are used outside the body.

The first two groups of substances are of important therapeutic interest as they contain products of comparatively low toxicity, some of these being pharmacologically almost inert, for example, penicillin. They offer, therefore, possibilities of wide application and will play an increasingly important rôle in the future.

THE PHARMACOLOGY OF SULPHONAMIDES

The formula of the parent compound is



Increased activity is sometimes achieved in derivatives formed by

altering the free amino or the sulphonamide group. These chemical derivatives, however, generally show the same characteristic reactions as the simple sulphonamide, which enables exact estimation in blood and body fluids.

All the compounds of the group exert a bacteriostatic effect through blockage of one of the enzyme systems in the bacterial cell essential for synthesis of growth factors (Wood, 1940). This action can be inhibited by providing the bacteria with the growth factors, such as para-aminobenzoic acid, which antagonises sulphonamides in the proportion of 1 part to 26,000 (weight); or to a less degree by adenine or methionine. Anaesthetics of the para-aminobenzoic group (procaine) also neutralize the inhibitive action of the drug. Sulphonamides which are absorbed circulate in the blood, diffuse through the body fluids such as bile, cerebrospinal fluid, and so reach the bacteria deposited in the infective lesion. The lowest effective concentration to act bacteriostatically is 2-3 mg per 100 cc. of blood, and this is the minimum level to be aimed at during treatment. The effective concentration in the blood and that at the focus of infection depend on the degree of absorption conditioned largely by the solubility of the substance, the rate of excretion in the urine, and by the conversion ratio of the free substance into less active acetylated forms (which are usually less soluble in the urine).

The introduction of a succinyl or guanidine group into the molecule decreases the rate and degree of absorption and such preparations are recommended for intestinal infections. The rate of excretion of the different compounds is governed by such factors as the solubility of the free and acetylated form in the urine at different pH and the reabsorption of the excreted compounds in the renal tubules. Sulphathiazole is, for instance, rapidly excreted and not reabsorbed in the renal tubules, whereas sulphadiazine and sulphamerazine are reabsorbed. The majority of these sulphonamides are more soluble in alkaline urine. The antibacterial activity of the compounds is also increased in an alkaline fluid and is affected by the portion of the compound circulating freely in the body in ionic form, and therefore dialyzable. Part of the compound is bound on the plasma protein and therefore not dialyzable; it is thus inactive. The percentage of bound compound varies, *e.g.* sulphadiazine 20 per cent., sulphapyridine 30 per cent., sulphamerazine 40 per cent., sulphathiazole 60 per cent.

The toxicity of different compounds depends on the blood concentration, the duration of treatment, and the ratio between the free and acetylated form or plasma bound fraction—in which case

there is some evidence that the latter may be the cause of sensitization. It may be difficult to differentiate toxic from sensitivity reactions. Direct toxic manifestations are: kidney disturbances, blood disorders, haemolytic anaemia and agranulocytosis, central nervous system disturbances (neuritis, epileptiform convulsions). Apart from these direct manifestations, indirect toxic symptoms may occur during prolonged treatment, e.g., secondary vitamin deficiencies caused by suppression of vitamin B producing bacteria in the intestine. The commonest allergic reactions are drug-rashes and drug-fever. Sensitization to the drug may show up unexpectedly if, after an interval, treatment is renewed with the same or some closely related compound. In this respect different substances behave differently and sulphathiazole seems to be more sensitizing than other sulphonamides. Granulocytopenia may also be a sign of sensitization and not damage of the bone marrow—in such a case sternal puncture may help in prognosis and treatment with penicillin should be begun to suppress secondary infection.

For full information on the medical uses of sulphonamides see M.R.C. War Memo. No. 10 (1945, 2nd Ed.).

THE PHARMACOLOGY OF PENICILLIN

Penicillin, an active antibacterial substance, is produced as a metabolic product during the growth of the mould *Penicillium notatum* on liquid medium. After a period of 7–9 days' growth at 24° C. the yellow-coloured solution is collected. Depending on different conditions the potency of the crude fluid varies widely from 10 to 150–200 units or over per cc, in which latter case the fluid can be applied without further purification for external use, as was originally suggested by the discoverer of penicillin (Fleming, 1929).

The purification of penicillin is carried out either by adsorption or solvent extraction, and the final product is kept dried in ampoules. Pure penicillin shows an activity of 1,650 units per mg. and forms colourless crystals which dissolve readily in water.

The potency of penicillin has been described in terms of the "Oxford" unit, a standard adopted by Florey and his co-workers, based upon the inhibitory effect of a minimal amount of penicillin on a strain of *Staphylococcus aureus* cultured in nutrient broth. More recently, an International Standard Penicillin has been adopted, and the international unit is defined as the specific penicillin activity contained in 0.6 microgram of the International Penicillin Standard (a specimen of pure crystalline sodium salt of penicillin ii (or G) kept at the National Institute for Medical Research, Hampstead); the international unit is approximately equivalent to the

"Oxford" unit. The test organism is a strain of *Staphylococcus aureus*, for practical purposes equally sensitive to Penicillin i (F) and ii (G).

Penicillin acts as a weak acid, the commercial product is stable but slightly deliquescent as a sodium salt; it is more stable as a calcium salt. Several variations of penicillin occur, possibly due to mutations of the mould, possibly to changes in nutritive conditions, or cultural conditions. Four varieties have been isolated in pure form, and distinguished by certain chemical characteristics; these are known in Britain as Pencillin i, ii, iii and iv and in America as F, G, X and K, respectively. These four penicillins may vary in their bactericidal activity against different bacteria *in vitro* while penicillin iv is quickly inactivated when in contact with body-tissues and therefore is of little value therapeutically. The International Penicillin Standard is a sample of the penicillin (ii or G) predominant in most existing preparations and most easily obtained as the pure salt in adequate quantities.

Penicillin is usually supplied as a powder; the commercial preparations at present available are not chemically pure but contain from 30 to 80 per cent. of known and also unidentified constituents both from the mould itself and from the media in which the penicillin was formed. Penicillin is not a protein; it is soluble in water; when kept in dry form in the refrigerator it remains stable for many months. Penicillin has a low toxicity; sodium penicillin, for example, is toxic to mice at the rate of not less than 3 g. per kg. body weight. In aqueous solution it is quickly absorbed from any place of injection and is excreted very rapidly in the urine; excretion commences about 15 minutes after an intramuscular injection—after 2 hours, 40–60 per cent. of the injected penicillin has been excreted. Penicillin is also excreted in other body fluids (e.g. bile) with the exception of the cerebrospinal fluid, in which it can be found only in the presence of inflammatory conditions, e.g. acute meningitis.

Oral treatment with penicillin is in equivalent dosage much less effective than parenteral therapy as much of the substance is destroyed in the gastro-intestinal tract; there have been suggestions that this difficulty might be overcome and oral therapy may become possible. Penicillin is active against mouth and throat infections when the substance is kept in the oral cavity in the form of pastilles or lozenges (McGregor and Long, 1944).

It is extremely important to handle penicillin solution aseptically, as many air-borne contaminants (e.g. *B. subtilis*) destroy it. It should be kept at a low temperature at pH 6.0–7.0. Penicillin is also easily destroyed when in contact with some metallic ions.

acids, alkalis, heat, oxidizing agents, and certain micro-organisms, particularly gram-negative "coliform" bacteria.

Fleming, in his original observations on penicillin, noted that the substance had a lytic effect on the staphylococcus, but following the work of Florey and his colleagues (1940) with the partially purified drug, emphasis has been laid on its bacteriostatic rather than its bactericidal action. However, recent investigations have confirmed Fleming's original observations and the bacteriolytic action of penicillin, particularly on young actively growing cultures has been demonstrated by different observers (Bigger, 1944; Todd, 1945). In some way, as yet undetermined, it interferes with cell-division, and it is therefore largely inactive against "resting" bacteria.

There is a fairly sharp cleavage among bacteria that are sensitive and those that are resistant to penicillin; in general gram-positive bacteria are sensitive and gram-negative organisms are resistant, but there are notable exceptions. The following list is a useful guide.

ORGANISMS SUSCEPTIBLE TO PENICILLIN	ORGANISMS NOT SUSCEPTIBLE TO PENICILLIN
<i>Strept. haemolyticus</i>	<i>Bact. typhosum</i>
<i>viridans</i> (variable)	<i>Salmonella paratyphi</i> (and other
<i>anaerobic</i> (variable)	<i>Salmonella</i>)
<i>Diplococcus pneumoniae</i>	<i>Shigella dysenteriae</i>
<i>Staphylococcus aureus</i>	<i>Bact. coli</i>
<i>albus</i>	<i>Bact. Friedländeri</i>
(some strains)	<i>Vibrio cholerae</i>
<i>Neisseria gonorrhoeae</i>	<i>Ps. pyocyanea</i>
<i>meningitidis</i>	<i>B. proteus vulgaris</i>
<i>Bacillus anthracis</i>	<i>Haemophilus influenzae</i> (except
<i>C. diphtheriae</i>	Pittman smooth strains)
<i>Actinomyces bovis</i> (variable)	<i>Haemophilus pertussis</i>
<i>Cl. botulinum</i>	<i>Brucella melitensis</i> (some)
<i>Cl. tetani</i>	<i>Monilia albicans</i>
<i>Cl. welchii</i> (<i>perfringens</i>)	<i>Streptococcus faecalis</i>
<i>Cl. septicum</i>	<i>Past. pestis</i>
<i>Streptobacillus moniliformis</i>	<i>Mycobacterium tuberculosis</i>
<i>Spirillum minus</i>	<i>Blastomyces</i>
<i>Leptospira icterohaemorrhagiae</i>	Viruses (except ornithosis)
<i>Treponema pallidum</i>	<i>Toxoplasma</i>
<i>Borrelia novyi</i> (relapsing fever)	<i>Plasmodium vivax</i>
	<i>Trypanosoma equiperdum</i>

The antibacterial action of penicillin is in general not appreciably affected by plasma, blood cells, pus or other known inhibitors of the sulphonamide compounds, *e.g.*, para-aminobenzoic acid, procaine. There is evidence to show that the sulphonamides and penicillin have a synergistic action against bacteria, subminimal doses of both substances, inactive individually, exerting a curative effect in combined treatment. Thus Bigger (1946) has shown that penicillin and sulphathiazole exert a synergistic action on *Bact. typhosum in vitro*.

TREATMENT WITH PENICILLIN

For the treatment of generalized infections penicillin may be injected by any parenteral route: intravenously, intramuscularly, subcutaneously, or into the bone marrow, either by the continuous drip method or by repeated injections every 3 hours. For more localized infections it has proved valuable to inject penicillin directly into the body cavities such as the peritoneal, pleural or joint cavities, as the penicillin is then less quickly absorbed, which permits a greater local concentration, less frequent injection, and therefore economy of the drug. Penicillin is also active when applied on infected surfaces, *e.g.*, burns and skin infections, in the form of dressings, using a weak solution, a powder, or an ointment (see M.R.C. War Memo. No. 12). Inhalation of finely atomised penicillin has been used in lung infections.

The total dosage of penicillin required for treatment depends on the type of infective organism, localization of the disease and the route of administration. In streptococcal septicaemia a minimum dose of about 100,000 Oxford units is given in 24 hours, and double this dose may be required in cases of staphylococcal septicaemia. If single injections are given, the common practice is to give a dose of 15,000–20,000 Oxford units intramuscularly in 1½–3 cc. of saline every 3 hours; the drip method, either intramuscular or intravenous, is favoured by many because it keeps the concentration of penicillin in the blood at a level sufficiently high to have continuous bacteriostatic effect. A blood level of 0.05–0.1 Oxford unit per cc., should be aimed at with the drip method; 100,000–150,000 units in intravenous drip over 24 hours gives an average blood level of about 0.1–0.14 Oxford unit/cc. In kidney disorders (nephritis) the blood concentration of penicillin is maintained at higher levels for a longer period. In septicæmic conditions, treatment with penicillin should be continued for

7-10 days with a total dosage at least 1,000,000 units. Staphylococcal infections are particularly liable to relapse if treatment is stopped too soon. For more localized infections, e.g. pneumonia, treatment need not be so continuous (4 injections may be given daily and none through the night) nor so long-continued; total dosage 200,000-400,000 units. In gonococcal infection, a total dose of 100,000-150,000 units given intramuscularly, distributed over 1-2 days will bring about a cure. Early syphilis responds to intensive treatment (2-2½ million units) over a period of 8 days, but relapses have been reported and it may be wise to use a combined penicillin and arsenical course.

In systemic infections it is advisable to give penicillin in high dosage, for the organisms may become penicillin-resistant if treatment with low doses is prolonged. As a general rule no case should be treated with penicillin unless the infective agent has been isolated and found to be penicillin-sensitive, if possible the degree of sensitivity should be ascertained. Progress of the treatment should be estimated by quantitative blood cultures, and penicillin blood levels. The blood culture often becomes sterile after one or two days' treatment, a combined effect of penicillin and the phagocytic action of leucocytes. The temperature chart may not be a reliable guide of the response to treatment as pyrexia may continue for some days despite obvious clinical improvement.

Certain reactions have been noted following the administration of penicillin. They are by no means constant and may differ from batch to batch of penicillin. Some degree of fever has been noted, and with the continuous intravenous drip, thrombosis of the vein may occur. There may be pain after intramuscular injection, a skin rash, urticaria and occasionally headaches, though with penicillin of greater purity, these should not occur. Cases have been reported of *pyocyaneus* infection at the site of injections where the strictest technique was not employed.

TESTS FOR SULPHONAMIDE SENSITIVITY OF BACTERIA

It is sometimes necessary to determine whether a strain isolated from a patient is sensitive to sulphonamide and to what degree. Three methods are available, and they are described below in order of suitability.

1. Plate Method: Blood agar plates, prepared from 3 per cent. nutrient agar, are poured so as to give a 3-4 mm depth of medium. Parallel gutters and ditches about 1 cm. wide are then cut across

the surface of the medium using a sterile scalpel, and the agar removed with a sterile platinum loop. Suitable dilutions (e.g., 2 mg., 5 mg., and 10 mg. per 100 cc. of medium) of the sulphonamide to be tested are prepared in melted blood agar and, by means of a sterile pipette, the gutters are filled until the surface of the medium is level. About 3 gutters, permitting 3 dilutions of the sulphonamide, can be cut across a 9 cm. Petri dish. After preparation, the plates are stored in the cold for 24 hours before use. The strains to be tested are grown in broth overnight, diluted 1 : 1,000 (roughly one loopful to 5 cc. of saline) and inoculated evenly as streaks across the plate at right angles to the gutter. The plates are then incubated overnight at 37°C and examined. Growth will be seen to have stopped short at a variable distance from the gutter if the strains are sensitive to the concentration of sulphonamide used. If the strain is resistant growth will be up to and across the gutter. Known sensitive and resistant strains should always be included in the test as controls.

The method described may give inconstant results owing to sulphonamide-inhibiting substances in the medium, while the gradual diffusion of sulphonamide from the gutter into the medium in the rest of the plate makes it impossible to say exactly what is the inhibiting concentration of the drug. Sulphonamide-inhibitory substance can be neutralized by using 5 per cent. *lysed horse blood* (blood from other species is unsuitable) in the preparation of the medium. The lysed blood is prepared by removal of the serum or plasma, substitution of an equal volume of sterile water, and freezing and thawing the mixture which then keeps indefinitely. strengths of 2 and 10 mg. of sulphanilamide, and 1 and 5 mg. sulphathiazole, in 100 cc. of nutrient agar are used, and as usual the test cultures are diluted 1 : 1,000 before being streaked over the plate. (For details, see M.R.C. War Memo. No. 10 : 2nd Ed. 1945.)

2. Slide Cell Technique : Preparation of the Slide Cells. Using paper of about 0.2 mm. thickness (Fleming recommends paper of 0.2 mm. thickness) cut 2.5 mm.

A mixture is sterilised by heating. When it has cooled to about 50°C. the paper slips (held in sterile forceps) are immersed in the fluid, withdrawn, and carefully laid on a sterile 3 × 1 inch slide, according to the pattern required—i.e. whether 4 or 6 compartments are to be formed. A further sterile slide is then warmed in the Bunsen flame and placed firmly but carefully on the top. (Prepared slides can be stored in

a box.) The preparation is then laid on a sterile sheet of glass measuring 10×20 cm. and is covered with the top of a sterile Petridish.

Using citrated sterile blood (human or animal) dilutions of the sulphonamide are made in test tubes, a control tube containing blood without sulphonamide being also prepared. A saline suspension of the strain to be tested is next prepared, containing about 100,000 organisms per cc., and one drop of this is added to 5 cc. of each dilution of sulphonamide in blood. The upper slide covering the cells is gently slid back (about 1 mm.) and the infected blood sulphonamide mixtures are then introduced into the cells with a capillary pipette. The fluid is drawn into the cells by capillary attraction; the upper slide is replaced so that the slides are in alignment. Using a sterile camel-hair brush the cells are sealed by painting along the open edge with the wax vaseline mixture. The completed preparation is then (for convenience) cemented to the sheet of glass by a thick layer of beeswax applied hot. Great care must be taken to ensure that the cells are completely sealed. The preparation is then incubated at 37°C . overnight and examined under low microscopic power for growth. In the case of organisms which produce haemolysin, growth may be estimated from the extent of haemolysis.

3. Tube Dilution Method: This method is best performed by using a synthetic or semi-synthetic medium from which any sulphonamide inhibitors are excluded. Dilutions of the sulphonamide are prepared by the serial dilution method in the medium itself. Generally it is most satisfactory to start with the first dilution as 1 : 1,000 of the sulphonamide (if the particular sulphonamide is not soluble, a sodium salt may be formed by adding the minimum of caustic soda solution to dissolve all the solid). Each of the tubes in the series is then seeded with a *minimal inoculum* of the tested organism from a 24-hour culture grown in the synthetic medium. The tubes are incubated at 37°C . Growth, recorded after 24 and 48 hours' incubation, is compared with that of the control tubes without sulphonamide. The specific inhibition of the sulphonamides can be proved by adding to the sulphonamide dilution traces of para-amino-benzoic acid (1 : 500,000) which will enable the tested organism to grow in presence of the sulphonamide.

Discs of blotting paper impregnated with 0.04 cc. of an 0.25 per cent. solution of sulphathiazole in broth and placed on the primary blood-agar plate after inoculation make a useful "screening" test for sulphonamide sensitivity of haemolytic streptococci, staphylococci, etc. (Morley, 1945).

TESTS FOR PENICILLIN CONTENT

Blood

It is important to test blood levels during treatment with penicillin, in order to indicate to the physician the efficiency of the doses used and the frequency of injections. The following methods are available:

1. **The Serial Dilution Method**, for which at least 2 cc. of serum are necessary. A series of dilutions are prepared in broth tubes beginning with 1:2 and doubling the dilutions. To each of the tubes is then added one drop of a 24-hour culture of a standard *Staphylococcus aureus* strain, incubated overnight at 37° C. Growth is recorded. If inhibition occurs in the dilution 1:50, the tested serum contains roughly 1 Oxford unit.

2. **The Micromethods of Fleming (1944)**. The advantage of these methods, in which a haemolytic streptococcus sensitive to penicillin is used as the test organism, is that only very small amounts of blood, obtainable by ear or finger-prick, are needed.

(a) **SLIDE CELL METHOD** Slide cells are prepared as described in the section on sulphonamides. Six compartments are used, each holding almost 50 cmm. As an indicator, human blood of group "O" is used, to which "Liquoid" is added up to a concentration of 1:1,000-1:2,000, to prevent clotting and to destroy the leucocytes. Defibrinated horse-blood gives equally satisfactory readings. One cc. of a 30 per cent. suspension of the defibrinated blood is inoculated with 1 loopful of a 24-hour broth culture of haemolytic streptococcus. Serial dilutions of the patient's serum are made on a paraffined slide, viz. a series of volumes of 25 cmm. of saline are placed on the slide, and a volume of 25 cmm. of the patient's serum is placed on the end of the slide and mixed with the first drop. A series of dilutions are prepared of 1:1, 1:2, 1:4, 1:8, 1:16, the last drop of saline serving as a control. Then 25 cmm. of the infected blood are mixed with each dilution, and the mixtures are run into the slide cells. The slides are incubated at 37° C. and examined horizontally by transmitted light. In the slides in which an effective concentration of penicillin is present, no change takes place; in the cells in which streptococci grow freely, the blood is completely haemolysed. If the tested blood contains 1 unit of penicillin per cc., inhibition occurs up to a dilution of 1:32, but with a biological test interpretation of inhibitory dilutions in terms of penicillin concentration can only be approxi-

mate. Ordinarily an inhibitory dilution of 1:2-1:4 represents an adequate concentration of penicillin in the blood.

(b) THE CAPILLARY TUBE METHOD is simpler to perform and results are more consistent. Glass tubes drawn to a calibre of about 0.8 mm and cut in 7.5 cm. lengths are sterilized and stored in sterile test tubes. The test organism and blood are prepared in

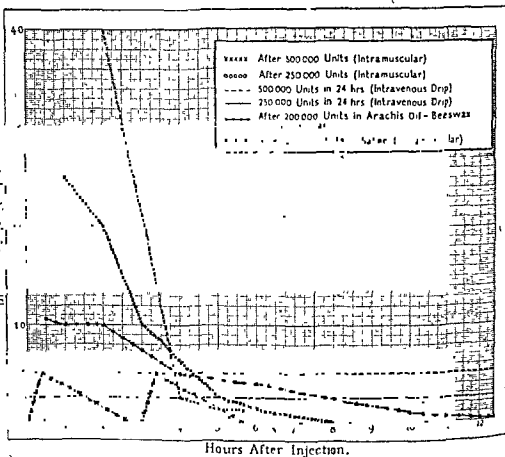


FIG. 2.

the same way as for the slide cell method. To obtain a good end-point, the final mixture in the test must not contain more than 10 per cent blood. Serial dilutions of the patient's serum (25 cmm.) are made on a paraffined slide as before. Each dilution is mixed with 5 cmm of the infected blood (diluted twice with saline). One by one the drops are touched with the end of one of the capillary tubes, held at a low angle so that the fluid runs up the tube. The tubes are tilted until the fluid reaches to about the middle of each; then the ends are sealed in the flame. The tubes, fixed horizontally

in plasticine, are incubated overnight at 37° C. and examined for signs of haemolysis. If the tubes are then set upright, the undissolved corpuscles settle to the bottom, and after 1 hour an end-point can easily be fixed.

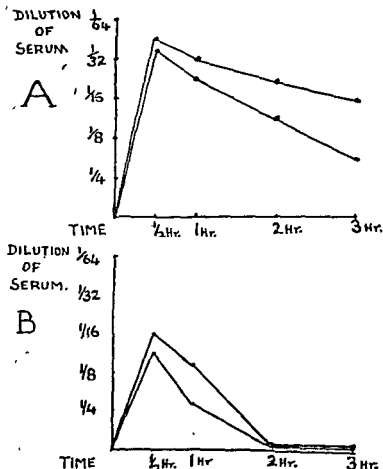


FIG. 3.—Rate of Excretion of Penicillin in 'A' Patient with Staphylococcal Septicaemia and Nephritis; 'B' Patient with Multiple Staphylococcal Abscesses without Renal Damage. Dosage 15,000 Units I M.

In performing the estimation it is important to know the time of the last injection of penicillin, so that dilutions of serum can be prepared in a suitable order. If, for example, the intramuscular injection was given more than an hour before the blood was taken, it is not necessary to prepare dilutions higher than 1:16. The serum should be tested on the day of withdrawal, for, if kept any

length of time, the penicillin level may drop. The optimal effective blood concentration of penicillin is about 0.1 Oxford unit per cc., although 0.02 Oxford unit per cc. is bacteriostatic. The charts show the dependence of blood levels on (a) time of injections and doses (Fig. 2), (b) conditions of kidney function (Fig. 3).

Pus and other Body Fluids

One method is to test on similar lines as for blood. If the pus is too thick to be tested it may be shaken with an equal part of broth, incubated for half an hour at 37° C., slightly centrifuged, and the supernatant fluid tested for penicillin content.

An alternative method is the use of the plate technique. Nutrient melted agar cooled to 50° C. is inoculated into a freshly prepared suspension of the test organism (*Staphylococcus aureus* or *B. subtilis*). Plates 3.4 mm. in thickness are poured and dried in the incubator. Heatley's cylinders (b) or sterile filter paper discs of 1 cm. diameter are placed on the medium, or holes of 1 cm. diameter are cut with a sterile cork borer in the agar and the bottom sealed with a drop of melted agar. A known volume of the fluid, e.g. 0.05-0.1 cc. is placed in the cup or hole or on the disc. On the same plate are placed known dilutions (two or three different levels, e.g. 0.2, 0.4, 0.8 Oxford units) of standard penicillin. The plates are then incubated overnight at 37° C. The zones of inhibition of the control penicillin dilutions are measured and a standard curve is set up, referring to the penicillin level and the zone of inhibition. On this curve, from the zone of inhibition, the unknown content of penicillin in the test fluid is determined. Specimens of urine may be diluted 1:5 for the test. If, as sometimes happens, the specimen contains a coliform penicillin-resistant organism, it should first be Seitz filtered unless the cup method is used. Alternatively, the urine may be heated at 60° C. for $\frac{1}{2}$ hour which kills the coliform organisms without appreciably affecting the penicillin.

Tests for Penicillin Sensitivity of Pathogenic Bacteria

This is performed to establish the dose of penicillin required in the treatment according to the sensitivity of the infective agent to penicillin. This can be done by using.

1. Heatley cups or filter paper discs or holes in the agar, measuring the zone of inhibition with 2 different levels of penicillin (e.g. 1 and 5 units) and comparing them with zones of inhibition of the standard staphylococcus strain (Fig. 4).

2. The serial dilution method in tubes Each one of a series of broth tubes containing decreasing amounts of penicillin (e.g. from 1 unit/cc. to 1/250 unit/cc) is inoculated with one drop of a 24-hour suspension of the test organism. A second series of broth tubes containing the same dilutions of penicillin are inoculated with a 24-hour suspension of a standard staphylococcus strain. After 18 hours' incubation, growth in the tubes is recorded and the degree of inhibition of the tested strain is compared with that of the

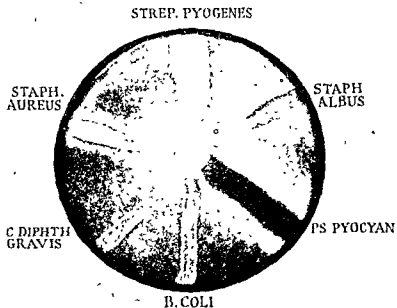


FIG. 4 —Central cup contains one unit of Penicillin.

standard strain. If the standard strain is inhibited by penicillin at a dilution of 1 : 60, and the tested organism at a dilution of 1 : 30, the tested organism is only half as sensitive to penicillin as the control.

Isolation of Bacteria from Specimens containing Penicillin

In the course of penicillin treatment it is sometimes desirable to determine if the infective organism is still present in the blood, or other body fluids. One cc. of blood is taken aseptically from the vein and mixed with 10 cc. of agar melted and cooled to 45° C.

Add one drop of penicillinase,¹ pour into a Petri plate, inoculate for 24 hours, and examine. The colonies, when counted, give roughly an indication of the number of bacteria in 1 cc. of blood. If a purely qualitative test is required to determine if bacteria are present, 3-5 cc. of blood is added to 50 cc. broth, to which a few drops of penicillinase have been added. For the bacteriological examination of other fluids that may contain penicillin, a few drops of penicillinase are spread on one half of a blood-agar plate, dried and the plate then inoculated with the fluid. With swabs from infected wounds, the addition of penicillinase is usually unnecessary as there is insufficient penicillin carried over to inhibit growth other than at the site where the swab has been rubbed on the plate.

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¹ Penicillinase is a relatively thermostabile enzyme secreted by some penicillin-resistant bacteria and specifically destructive to penicillin. It can be prepared by growing *B. subtilis* in 20-25 cc. of digest broth in a 100 cc. container for 3 days at 37° C. Potency is enhanced by adding 500-1,000 units of penicillin during the second or third day of growth. The fluid is adjusted to pH 7.2 and Seitz filtered. The potency of penicillinase varies with different strains of *B. subtilis*, 1 cc. of a 1:250 dilution of a good filtrate will inhibit 50 units of penicillin in 1-2 hours at 37° C (Duthie, 1944; Ungar, 1944).

CHAPTER XII

LABORATORY DIAGNOSIS OF LEPTOSPIRAL INFECTIONS

THE commonest of the leptospiral infections in Europe is Weil's disease, caused by *Leptospira icterohaemorrhagiae*; less frequently and in more restricted areas have been found infection by *L. canicola*, reported chiefly in Holland and Germany, and by *L. grippotyphosa*, which causes swamp-fever, etc. in Central and Eastern Europe. Weil's disease was separated from other forms of jaundice on clinical grounds in 1886, but diagnosis of it was very uncertain until Inada and others demonstrated in 1915 that the spirochaete which is now named *L. icterohaemorrhagiae* is the cause of the disease. This discovery was made in Japan; Germans independently came to the same conclusion and British workers confirmed the claims in observations made in the trenches of the Western front. Since that time the disease has been studied all over the world and recognised in all well-populated regions except in China, where investigation has not been thorough, and in India, Persia, Arabia and Egypt where it is possible that the strong sunlight may be lethal to the leptospira.

So far as is known, the natural habitat of *L. icterohaemorrhagiae* in a form virulent to human beings, is the kidneys of rodents, especially rats, which may harbour the organism for most of their existence with very little effect except the production of antibodies in the blood. The leptospirae are excreted constantly in the urine of these animals and contact with rats' urine is the most frequent cause of infection in man, or in dogs or foxes. The chance of invasion by the organism is increased by wet conditions, which delays the death of the leptospira and makes the human skin sodden, and by abrasions of the skin. These facts and the general epidemiology of the disease (Alston and Brown, 1937) are important in diagnosis since it is uncommon for the disease to be found unless occupation or other circumstances has brought the patient into a situation where rats are known or likely to be present. There are records of 274 instances of the infection in Great Britain from July 1933 to July 1939; 225 of these were in men and women who worked in rat-infested fish-gutting sheds, of coal miners in "wet" pits, of sewer-workers or of people who, within the incubation period of 4-19 days had been bathing in fresh water in places where rats

were known or likely to infest the ground at the edge of the water; all of the remainder of the 274 patients were people (almost all men) whose work was in places frequented by rats—tripe-scrappers, a slaughterhouseman, farm workers, gardeners, a zoo-keeper, a canal-worker, a gravel-pit worker, a water-cress grower, pipe-layers, or people who had been bitten by a rat (in seven cases) or by a dog or a ferret which had just caught a rat. Another aspect of the relation of infection to contact with rats is that between 1933 and 1939, 40 out of 46 infections in London were in sewer-men, and similarly in Aberdeen, 98 out of 102 were in fish-workers in unhygienic premises.

It is obviously in the more severe instances of the infection that diagnosis and the appropriate treatment are most urgent but, as in other infectious diseases, every degree of severity of the infection can be recognised. For example, during the 1914-18 war, as many as 60 per cent. of patients had a mild illness without jaundice; similarly, among 1,000 men who work regularly in the London sewers, 34 had the illness seriously enough during the years 1934-1938 to be treated in hospital and, of these, 6 (18 per cent.) died; but mild infections, also, which did not necessitate admission to hospital were found, and of 45 healthy sewer workers who had never had jaundice, 9 showed significant agglutinins and protective antibodies.

LABORATORY DIAGNOSIS

Accurate diagnosis depends on demonstrating the leptospira, by guinea-pig inoculation (or less certainly in sections of the patient's tissues) or by agglutination test of the patient's serum, cerebrospinal fluid or an effusion in a body cavity. These means should always be carried out in suspected cases for guiding treatment and also because the disease has been scheduled in Great Britain under the Workmen's Compensation Act, 1925, with the special proviso that confirmation by bacteriological or serological examination is required. While these tests are being made, clinical observations and simpler pathological investigations are valuable.

In countries where yellow fever, spirochaetal relapsing fever, dengue and blackwater fever need not be considered, the conditions which require to be distinguished from Weil's disease or other forms of leptospiral infection are febrile illnesses in which jaundice has occurred as an unusual complication, and the frequent form of jaundice which may be referred to as infective hepatitis. Severe muscular pains at an early stage of an illness with fever and jaundice

suggest Weil's disease as does albuminuria or more definite evidence of acute nephritis such as casts and erythrocytes in the urine. A leucocyte count should always be made as one of the earliest steps in pathological diagnosis, because it helps very greatly in distinguishing infective hepatitis (which is accompanied by a leucopaenia with relative or absolute lymphocytosis) from most of the other conditions mentioned above, which are usually accompanied by a polymorph increase. Weil's disease shows almost always a leucocytosis of 10,000-30,000 cells per cc. with polymorphs comprising 70-95 per cent. It must be remembered, however, that such conditions as streptococcal septicaemia with jaundice and carcinoma of the head of the pancreas with portal pyaemia have given similar leucocyte counts and clinical appearances which closely resembled those of Weil's disease.

The exact laboratory diagnosis depends on the fact that the leptospira may be demonstrated in the blood during the first week of the illness and in the urine during the subsequent week or fortnight and that antibodies may be found in the serum in significant titre from about the fifth day.

Animal Inoculation : Microscopical demonstration of the leptospira in human blood by dark-ground illumination is very rarely successful. Of the diseases mentioned above, it is only in spirochaetal relapsing fever that the causative organism *Spirochaeta recurrentis* may be seen readily in the patient's blood. Similarly, direct culture of human blood in artificial media is not usually successful. The isolation of the leptospira from the blood requires that clotted blood—both serum and clot—be inoculated into guinea-pigs as soon as possible after the withdrawal; rubbing some of the blood clot into a large area of shaved and slightly scarified skin on the animal's abdomen is a very effective route of inoculation; if the blood is free from secondary infection or contamination it is useful to inject further serum and broken clot subcutaneously and intraperitoneally; and, in these circumstances up to 3 cc. of blood may usefully be injected into each animal. If intraperitoneal inoculation has been done, an abdominal paracentesis on the 4th to 6th day after injection may show active leptospirae in sufficient numbers to make their identification certain, but if a positive agglutination test of the patient's serum has already confirmed the clinical diagnosis it may be preferable to wait until the animal dies or is moribund about the 9th to 12th day. The guinea-pig usually shows an increase of temperature (above 102° F., which is within the normal range) from the 5th day after injection, but no other

outward sign of infection occurs until roughening of the coat, decrease of activity and jaundice (seen best on the ears) are found on the 8th to 10th day, 12-48 hours before death. At post-mortem examination haemorrhages may be found in any tissue and jaundice in almost all except the nervous system, with congestion and cloudy swelling of the glandular organs. Blood in wet preparation by dark ground microscopy will show leptospirae as slender, cylindrical, highly flexible filaments 6-15 μ in length with tightly wound, small spirals. The extremities may be bent over in the form of a hook and owing to rapid rotation of the whole organism on its long axis it either moves rapidly among the corpuscles of the specimen or, if both ends are flexed, remains spinning rapidly in one area. If a leptospira is kept in view for a few minutes the strong light decreases its rotation and the fine, even coils, appearing as close as the strands of a rope, can be seen more easily and the organism distinguished from the blood threads which may be confused with it. This demonstration of leptospirae in a guinea-pig showing jaundice and haemorrhages after injection of blood from a patient is usually considered final for diagnosis and it may be noted that material from patients suffering from yellow fever, spirochaetal relapsing fever, dengue, blackwater fever and infective hepatitis do not cause jaundice or multiple haemorrhages in guinea-pigs. Sometimes the blood from a patient with Weil's disease fails to kill a guinea-pig or to reveal severe infection or discoverable leptospira and yet inoculation of this animal's blood and kidney, liver and suprarenal tissue into another guinea-pig will produce a fatal infection with the usual signs. The leptospira may be stained by silver methods such as Dobell's in sections of kidney, suprarenal gland or liver and may be isolated from minced portions of these tissues or the blood if no contaminating organisms are present. These cultures can be grown at 30° C. in simple fluid media such as Fletcher's (Appendix) or Schuffner's (1934); the organism has not yet been grown on the surface of solid media.

After the first week of the illness the leptospirae are less likely to be found in the patient's blood and may be sought by similar procedures in the urine. For this it is very advisable to make the patient's urine alkaline, as the leptospirae are quickly damaged by acid urine. Inoculation should be done as quickly as possible after it has been passed and after adding saponin in the proportion of 1 c.c. of urine and centrifuging at high speed; the leptospirae are present in large numbers in the urine. Meningitis is the most prominent evidence of

Weil's disease and the organism may be sought in the cerebrospinal fluid in these cases as well as in the blood and urine.

Agglutination Test : The patient's serum shows antibodies in significant concentration from about the 5th day of the illness. It is unfortunate that stable suspensions of the leptospira for agglutination tests cannot be prepared with certainty and careful controls of the state of the antigen as to its response to serum known to contain agglutinins and its insensitiveness to negative sera must be made. It may happen that the culture of a particular strain of *L. icterohaemorrhagiae* (often one which has lost its virulence for guinea-pigs) will supply a satisfactory suspension for a long time, but suddenly lose its agglutinability or become clumped in culture, in saline or in known negative sera. It is for this reason that the serum tests have not yet become usual practice in most bacteriological laboratories. Suitable cultures in Fletcher's broth are killed by adding formalin neutralized with pyridine (Appendix) Following Schüffner, it has been a frequent practice to make up the mixtures of patient's serum and antigen in small amounts in the cups of an artist's palette, commencing with two dilutions of 1 in 10 and 1 in 30 and making a series of dilutions of 1 in 100, 1 in 300 and so on up to 1 in 10,000 and 1 in 30,000. Controls with known positive and negative sera are desirable. Leave the test at room temperature or at 30° C. overnight and read by examining drops by the dark-ground microscope.

In subjects unlikely on account of their occupation to have been previously infected agglutination at a titre of 1 in 300 may be regarded as diagnostic. A low titre of agglutinins and doubt as to exact onset of infection are indications for repetition of the test after the lapse of a few days. Recent infections will show a steep rise of titre. The therapeutic administration of anti-serum may bring about the appearance of agglutinins in the patient's serum, but even with maximum dosage (see below) these will never reach a titre of over 1 in 500 and will disappear within 72 hours of their introduction.

Prognosis

In several series of hospital cases the mortality has been between 15 and 20 per cent. For prognosis, tests of renal function are useful; in most fatal cases nephritis appears to be the determining factor. Heavy albuminuria with many casts and oliguria are of bad significance; the blood urea gives a fair indication of the progress of the case; in most cases with infection severe enough to

demand admission to hospital the blood urea will reach 100 mgm. per 100 cc. ; rise to 250 mgm. need not occasion undue anxiety ; figures above this are of increasingly serious import. In infective hepatitis the blood urea remains at normal levels

Post-mortem Diagnosis

At post-mortem examination, blood and portions of kidney, liver and suprarenal gland should be taken for inoculation of guinea-pigs (scarification is the best method if material is contaminated) and for sections and, if blood suitable for a serological test cannot be obtained, cerebrospinal fluid or an exudate from a serous cavity may be tested instead, although the agglutination reaction with these fluids is likely to be weaker than with serum or even negative in a patient whose blood is positive.

Treatment

An antiserum is available (Burroughs, Wellcome & Co.) containing agglutinins and lysins against *L. icterohaemorrhagiae* which protects guinea-pigs against infection ; it is advocated in doses of 40 cc. per day until the temperature is normal ; there are no records of series of patients treated with and without the serum for appraisal of its value. Similarly, penicillin has been shown in the United States and in this country to kill the leptospira *in vitro* and when relatively large doses are given to cure infections in guinea-pigs. Records of its use in the disease (up to 150,000 units per day for 10 days) are beginning to accumulate. In patients who are seriously ill it is desirable, I believe, to give both serum and penicillin.

Other Leptospirae

L. canicola resembles the leptospira of Weil's disease in form and cultural requirements and can be distinguished from it serologically and by producing a fatal infection in golden hamsters but no more than a transient illness without jaundice in guinea-pigs. The reservoir of infection is dogs which have been found in some countries to discharge it in the urine in connection with a mild or fatal illness or in a symptomless state. Adults and children have been infected in the home and in the course of veterinary work ; the disease is mildly febrile, usually without jaundice, resembling influenza, undulant fever or tuberculosis

L. grippo-typhosa and other types (*L. pomona*, *L. sejro*, etc.) have been found to be the cause of febrile illness contracted in different circumstances in many parts of Central and Eastern Europe and variously named summer or harvest fever, slime or mud-fever, swineherd's fever, etc. Three instances have been reported in soldiers of the British Army in France (Buckland and Stuart, 1945). The diagnosis can be made by agglutination and the organisms have been found in mice, swine and other animals, and in water contaminated by them

Appendix

Fletcher's Broth for Leptospira: For each tube of medium add 0.5 cc of Lemco broth (pH 7.4) to 3.0 cc of glass distilled water and sterilise in the autoclave, add 0.25 cc of rabbit serum recently obtained, inactivated by heating at 56° C for half an hour and passed through a Seitz filter. Incubate the tubes at 37° C, for 24 hours to test their sterility. Incubation for growth of leptospira should be at 30° C. Growth is maximum usually in 3-7 days

Formalised Suspension for Agglutination Tests: Select cultures in which there is no clumping of the leptospira when examined under the microscope and to each 10 cc of culture add 0.2 cc. of the following mixture—

Formalin, (commercial)	50 cc.
Pyridine	10 cc.
Water	150 cc.

Fletcher's Agar: To each 3 cc of Fletcher's broth add 0.1 cc, of ordinary Lemco agar. This very small amount of agar often gives growth of leptospira from blood cultures, etc., but these cultures are not suitable for preparing suspensions for agglutination.

J. M. ALSTON.

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CHAPTER XIII

LABORATORY TESTS IN VENEREAL DISEASE

1. THE GONOCOCCAL COMPLEMENT FIXATION TEST

THE complement fixation test has become established in many laboratories as a useful aid in the diagnosis of gonorrhoea and its complications, and as a criterion of cure of the infection. For a review of the literature together with details of technique and clinical application, the reader is referred particularly to the brief monograph by Price (1933).

Preparation of the Antigen

Freshly isolated strains of gonococcus or strains that have been dried from primary culture (see Appendix) are used, and it is advisable to mix 3-5 such strains together for the inoculation of the medium from which the antigen is to be prepared. Price's egg medium (see Appendix) or any good "digest" medium to which 10-20 per cent ascitic or hydrocele fluid is added gives quick luxuriant growth that makes a good antigen. The medium is prepared in flat bottles such as 20 oz. "medical flats" with screw-cap as supplied by United Glass Bottle Co. or the triangular Roux bottles; these are inoculated with a saline suspension of the gonococcus culture by means of a Pasteur pipette. After incubation for 24-48 hours (depending on the rate and amount of growth) the gonococcal culture is washed off with 100 cc. normal (0.9 per cent.) saline and transferred to a 100 cc. cylinder. It is standardized at this stage to an opacity equivalent to 500 million organisms per cc. (approx.). One cc. of N/1 NaOH is then added to every 100 cc. of the standardized gonococcal suspension and the cylinder is placed in the water-bath at 37° C. until the suspension has gone into solution ($\frac{1}{2}$ -2 hours). The fluid may then be filtered through sterile lint to remove pieces of medium and other gross insoluble matter. To the clear filtrate 1.5 cc. of N/1 HCl is now added, and the cylinder returned to the bath. After 15-30 minutes, white flocculi appear; these are precipitated by centrifugation (3,000 r.p.m.) and then suspended in 4 cc. of sterile saline. Two drops of phenol red are added and N/10 NaOH drop by drop until a pH of 7.5 is reached. On vigorous shaking, the suspension

appears to go into solution. No further addition of alkali affects the solubility of the precipitate, but its antigenic properties become definitely weaker if the alkaline reaction is carried above pH 9.5, 1 cc. of 1 per cent formol-saline is added and the "solution" is then filtered through sterile lint. This colloidal solution constitutes the concentrated "antigen-reagent" which in the test proper is used in the appropriate dilution. At this stage the solution is heated at 60° C. for 1 hour, and then stored in the cold or at room temperature. During use it must be kept slightly alkaline as indicated by the faint pink colour of the indicator.

The antigen is titrated as follows: two rows of tubes (6 in the front and 10 in the back row) are placed in a rack. Serial dilutions of antigen are made from 1/10 to 1/100, and one volume of each dilution is placed in each of the appropriate pair of tubes. To each tube in the back row one volume of a known strongly positive serum, and to each tube in the front row one volume of a known normal serum is added. All the tubes then receive one volume of 3 minimal haemolytic doses of complement, the rack is well shaken, and placed in the 37° C. water-bath for 1 hour. At the end of this time one volume of "sensitized sheep" blood cells is added to all the tubes, the rack is again thoroughly shaken, and returned to the bath. After 30 minutes the antigen titre is read as half that dilution of antigen which just fails to fix 3 m.h.d. of complement in the presence of a normal serum. Thus if in the front row there is partial haemolysis in the first tube (1 : 10) and complete haemolysis in the second (1 : 20) the appropriate dilution for use is taken as 1 : 30 (double 1 : 15). A good antigen should in the presence of a positive serum completely fix 3 m.h.d. of complement in dilutions up to 1 : 100 so that all the tubes in the back row show "no haemolysis."

The Complement Fixation Test

Three 7.5 × 0.3 cm ($3 \times \frac{3}{8}$ in) tubes are used for each test which is carried out in a manner similar to the Wassermann reaction. A unit volume of each reagent is used (0.1 cc. is a suitable amount: or the dropper technique may be used).

Antigen The antigen is diluted in normal saline to the appropriate strength on the day of the tests but diluted antigen from the previous week may be used.

Patient's Serum : The serum should be taken off the clot within 24 hours of collection of the specimen of blood. It is used neat in

the gonococcal complement fixation test; and, is inactivated by heating at 58°C . for 20 minutes. (If the serum has been previously inactivated at $54\text{--}55^{\circ}\text{C}$. for the Wassermann reaction, it should be re-inactivated at the higher temperature to reduce the risk of anti-complementary effect.) Known negative and positive sera are included as controls

Complement Fresh guinea-pig serum is titrated as for the Wassermann reaction; doses of 3 and 5 m.h.d. are used for the test proper with 3 m.h.d. for the serum-control.

After these three reagents have been added in equal volumes to tubes 1 and 2 with one volume of saline instead of antigen in the third tube (serum-control), the tubes are well shaken and incubated at 37°C . either in the incubator for one hour or in the water-bath for half an hour. One volume of a suspension of sensitized sheep cells is then added to all tubes which are further incubated *until the cells in the known negative and serum control tubes are completely haemolysed*. This may occur in 15–20 minutes in the water-bath or in 30–40 minutes in the incubator. The tubes are then removed, allowed to stand at room-temperature for half an hour and the results read as follows:

Complete, almost complete, or marked trace of haemolysis in first tube: complete haemolysis in 2nd tube = **NEGATIVE**.

Trace or faint trace of haemolysis in first tube: complete haemolysis in second tube = **DOUBTFUL**.

No haemolysis in first tube: complete haemolysis in second tube = **WEAK POSITIVE**

No haemolysis in first tube: none or partial haemolysis in second tube = **POSITIVE**.

Clinical Application

A weak positive or positive reaction according to the above notation means, with rare exceptions, active or latent gonococcal infection. A negative reaction may occur with gonococcal infection under the following conditions:

(1) In the first week or two of primary anterior urethritis in the male; urethritis or early cervicitis in the adult female, and early vulvo-vaginitis in the female child.

(2) A patient with chronic gonorrhoea, in whom a local focus, e.g. vesiculitis, has after digital massage been drained, may give a negative reaction (after previous positive tests) although the focus is still infective.

(3) Some patients fail to produce antibodies in response to

gonococcal infection and this may also happen where a "closed" focus has formed.

In general the occurrence of a positive complement fixation test depends on (a) the duration of the infection 20-40 per cent. of acute infections will be positive by the end of the second week, 80-100 per cent. by the 4th-6th week; (b) the anatomical spread of the disease: extension to the posterior urethra, usually associated with infection of the seminal vesicles, quickly converts a negative to a positive reaction in most male patients. Again the occurrence of gonococcal Bartholinitis (the infection is frequently due to other organisms), or extension of infection to the upper cervical glands or to the Fallopian tubes is accompanied by a definitely positive reaction; (c) latent infection in the form of some closed focus, e.g. vesiculitis or salpingitis, is shown by a persistently positive reaction although the patient shows no signs of infection. (d) it is surprising how frequently arthritis and other "rheumatic" manifestations are associated with a positive gonococcal complement fixation test and this test should always be done on patients with such symptoms; (e) the use of vaccines for the treatment of gonococcal infection will temporarily enhance the gonococcal complement fixation reaction but a positive reaction due to vaccine-therapy does not usually persist for more than 6 weeks

False positive reactions may occur with meningococcal infections if the serum from the affected patient is tested within a few weeks of the attack (Cruickshank, 1941) Chronic nasopharyngeal infections in which *M. catarrhalis* is involved may also give rise to a weak positive complement fixation reaction with the gonococcal antigen (Price, 1933).

The test is useful as a criterion of cure although it may remain positive for some months after the patient has ceased to be infective. If the degree of positivity remains unaltered over a longer period, a persistent focus of infection should be suspected even if there are no clinical signs. When such a focus has been discovered and drained the complement fixation test usually becomes negative fairly quickly.

Appendix

Price's Egg White Solution.

Egg Albumen (B.D.H.)	.	.	.	100 g.
N/1 NaOH	.	.	.	150 cc.
N/1 HCl	.	.	.	60-70 cc.

Dissolve the egg albumen in 500 cc. distilled water overnight. Next morning add the NaOH, which will jelly the albumen, and place in the steamer for 2-3 hours. A slight precipitate forms and a clear ammoniacal smelling liquid results. This is filtered through Chardin paper whilst hot, and its pH adjusted to 7.5 with 60-70 cc. of N/1 HCl. A white precipitate may form, but this redissolves on thorough mixing.

Bottle in 25 cc lots and sterilize by free steam for 1 hour.

For use add 25 cc to 100 cc. of pH 7.5 Hartley broth agar.

Preservation of Organisms. Small strips of filter paper are cut, placed in Wassermann tubes which are plugged with cotton wool and sterilized. The organism is grown on blood-agar slopes, washed off with 2 or 3 drops of citrated blood (source—animal or human, immaterial) and the strip of filter paper extracted with sterile forceps, dipped into the blood-organism suspension and returned to the Wassermann tube.

The tubes containing the inoculated filter paper strips are put in a jar with some calcium chloride and the jar exhausted with a vacuum pump. The paper dries almost immediately and after 24 hours the tubes can be stored in a tin with some calcium chloride. The cultures remain alive for at least seven years. When required the filter paper is dropped into a tube of broth and incubated. E. R. JONES

(Personal Communication).

An alternative method using strips of thin waterproof cellophane has been described by Rayner [*J. Path Bact.* 55. (1943) 373]. See also *Handbook of Practical Bacteriology* by T. J. Mackie & J. E. McCartney (Edinburgh, 1945, 7th edit., p 666).

R. CRUICKSHANK.

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PRICE, I N O *The Complement Fixation Test for Gonorrhoea*. London (1933) (price 6d)

2. A SLIDE TEST FOR THE SEROLOGICAL DIAGNOSIS OF SYPHILIS

LAUGHLIN (1935) described a modified Kahn antigen which could be used for rapid diagnosis of syphilis by mixing the suspected serum and the antigen on a glass slide and observing the presence or absence of precipitation by the naked eye. The reliability of the test has been increased by further modification and titration of the antigen (Hamilton-Paterson *et al*, 1944), so that it shows a sensitivity (98.68 per cent.) greater than the Wassermann Reaction and

Kahn although its specificity (99.5 per cent.) is rather less than that of the latter tests. It is not claimed that the slide test should supplant those already established for the routine diagnosis of syphilis but it can be of great use in the preliminary sorting out of large numbers of sera such as are received from ante-natal clinics and blood banks

Preparation of the Antigen

Scrupulous cleanliness of all glassware is essential

REAGENTS.

(1) Kahn standard antigen (Kahn, 1925) This must have been titrated for the Kahn test and should give values between $1 \div 0.8$ and $1 \div 1.0$. Antigens which require more than 1.0 cc. of saline for sensitization have proved unsuitable for the slide test.

(2) Colloidal benzoin. Add 0.3 cc. of a 10 per cent. solution of crude Sumatra benzoin in absolute alcohol to 20 cc. of distilled water heated to 37°C , the mixture being shaken continuously. Filter through Whatman No. 41 immediately before use.

(3) 10 per cent. NaCl in distilled water. This is best freshly prepared.

(4) Sterile normal horse serum.

Set up six tubes $3 \times \frac{3}{4}$ in. in two rows of three. In the back row pipette 1 cc. of Kahn antigen to the bottom of each tube and in the front row 1 cc. of colloidal benzoin. Heat the tubes at 56°C . for 5 minutes. Mix the benzoin and Kahn antigen in each pair of tubes by pouring the mixture backwards and forwards six times without waiting for the tube to drain. Set up three more tubes and pipette into the first 1 cc. 10 per cent. saline, into the second 1.0 cc. 6 per cent. saline and into the third 1 cc. 6 per cent. saline. Mix the saline in each of these tubes by six pourings as already described with one of the tubes of benzoin-Kahn mixture and allow to stand overnight. Next day shake the three antigens and test them simultaneously with a strongly positive, weakly positive and three or four negative sera as described later. The accompanying table shows the results obtained from such a titration and the antigen choice is the one in which the positive sera give their full value and the negative sera show the least granularity, *i.e.* the antigen containing 8 per cent. saline in the table.

Now divide the selected antigen into two parts and to the first add one-third of its volume, and to the second one half of its volume of sterile normal horse serum. Shake the tubes and after standing

for 2 hours test both the antigens against positive and negative sera. The results are shown on the right-hand side of the table where it will be seen that 33 per cent. of serum was required to eliminate the non-specific reaction obtained with one of the negative sera. The formula of the antigen is now complete and can be marked on the Kahn bottle and no further titration is needed, until a fresh batch of Kahn antigen is used. In making the slide test antigen from the formula the serum is added 2 hours after the Kahn, benzoin and saline have been mixed and the completed antigen retains its sensitivity for periods of 14-21 days.

TABLE

Serum	Antigen			W R	Kahn	Antigen 8 % saline	
	1 10 % NaCl	2 8 % NaCl	3 6 % NaCl			25 % serum	33 % serum
1	++	++	++	++	++	++	++
2	+	+	±	+	+	+	+
3	G	S G	S G	—	—	S G	—
4	G	S.G	—	—	—	—	—
5	—	—	—	—	—	—	—

++ = strongly positive

+ = positive

± = weakly positive

G = granular.

S G = slight granularity.

The Test

No special apparatus is required. Deliver serum and antigen on to a slide with Pasteur pipettes which will drop approximately equal volumes. The sera should be previously inactivated at 56° C for 30 minutes. Stir the mixture with a glass rod taking care not to spread the fluid as this accelerates drying with the appearance of granularity in the negative sera. Rock the slide gently two or three times and put aside. To read the result rock the slide again and view against a black background with an oblique slide, holding the slide just above and in front of the slot of a microscope lamp and looking down through it on to a piece of black paper. For a strongly positive (++) reaction there is a marked precipitation and flocculation of the antigen with a complete clearing of the turbidity. In a positive (+) reaction the flocculation is less marked and there is only partial clearing. For a weakly positive (±) reaction there is

often flocculation but no clearing of the mixture. The negative should remain opalescent and show no granularity.

The time between mixing and reading is decided by first testing the antigen with a known negative, a weakly positive and a strongly positive serum. The first reading is taken after the strongly positive serum has given a complete reaction. This may vary between 3 and 5 minutes. The weakly positive serum may show some change in this time but may not give a complete reaction for a further 3 to 5 minutes. All the tests are read again after this time.

J. L. HAMILTON PATERSON.

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SECTION II

BIOCHEMISTRY

In accordance with the usage of the Chemical and Biochemical Societies, in this section the abbreviation ml. (instead of cc.) is used as the measure of Volume and μg (instead of γ) as the abbreviation for micrograms.

CHAPTER XIV

LIVER FUNCTION TESTS

THIS review discusses the application of liver function tests to practical clinical problems.

There are three reasons for doing a liver function test :

1. To assist in the differential diagnosis of jaundice with the chief object of distinguishing between "surgical" and "medical" cases.
2. To obtain confirmatory diagnostic evidence in support of clinically suspected liver disease.
3. To detect hepatic insufficiency, as an aid in prognosis and in directing medical treatment, particularly before and after operation.

It is important to choose a test which is suitable for the purpose. Adopting Ivy and Roth's (1943) clinical classification of liver function tests, a modified selection will be given according to the clinical problems to be answered.

1. Tests Useful in the Differential Diagnosis of Jaundice :

- a. Urobilinogen determinations in urine and faeces.
- b. Serial serum bilirubin levels.
- c. Serum colloidal gold reaction.
- d. Thymol turbidity test.
- e. Serum alkaline phosphatase
- f. Galactose tests.
- g. Duodeno-biliary drainage.

2. Tests Useful in the Detection of Hepatic Insufficiency :

- A. *To detect the extent of liver damage and residual function, to follow the course of liver disease and to assess prognosis and efficacy of treatment.*
 - a. Hippuric acid test.
 - b. Bromsulphalein clearance test.
 - c. Plasma prothrombin level and its response to vitamin K treatment.
 - d. Serum proteins.
 - e. Galactose tests.

B. To detect and follow the activity of hepatic disease.

- a. Serum colloidal gold reaction.
- b. Thymol turbidity test.
- c. Cephalin cholesterol flocculation test.
- d. Takata-Ara test.

The tests listed under 2A, and the qualitative urobilinogen test, in a limited way, may be used in support of the diagnosis of suspected "latent" liver disease.

THE INTERPRETATION OF LIVER FUNCTION TESTS**Limitations**

The problem of testing hepatic function has not been satisfactorily solved, and in view of the many limitations it is difficult to write of the positive aspects without many qualifications; although there is hardly one test which is entirely free from theoretical or practical objections, some of them have proved of clinical value.

Most liver function tests by themselves do not establish a clinical diagnosis, but, at best, answer one question only such as the presence of hepatic insufficiency or dysfunction, the clinical significance of which is largely a matter of interpretation by the physician. Whether the answer provided by the test will help to solve the clinical problem will depend not only on the suitability of the test for the purpose, but also on the constellation of clinical features in a given case. For example, a positive hippuric acid test is not by itself diagnostic of hepatic cirrhosis in a patient with vague dyspepsia admitted with haematemesis or melaena, since the same finding may be obtained, especially when the general condition is poor in uncomplicated gastroduodenal ulcer and in carcinoma of the stomach. A positive colloidal gold reaction would be more helpful in supporting the diagnosis of cirrhosis, while should the patient prove to have an ulcer requiring partial gastrectomy, the hippuric acid test would be useful in assessing the surgical risk. Even the most "specific" test of liver function is not absolutely diagnostic of "liver disease" in the strict sense, as hepatic function may be altered as the result of other disease processes, such as infection. But if these can be excluded clinically, less "specific" tests may be used in support of the diagnosis of latent hepatic disease.

"Dissociation" and "Correlation" of Liver Functions

The liver has a large number of functional activities which are not always injured at the same time or to the same degree. In a

given case one test may be positive indicating impaired function, while other tests may be negative implying normal function or even function above normal. In view of this "dissociation" it is obviously desirable to use, if possible, more than one test, preferably covering different activities in order to detect hepatic dysfunction. The fact that different disease processes give different patterns of disturbed function assists in differential diagnosis.

The "dissociation" and "multiplicity" of liver function tests should be kept in mind in interpreting negative results, for they do not exclude potential damage of the liver, which has great power of reserve and regeneration, but more emphasis may be placed on a "positive" result. It is not always possible or desirable to perform a large number of tests, and our present clinical approach to the problem of gauging "liver function" is based on the experience that positive results with one or a few reliable tests indicate that other functions of the liver are also likely to be impaired. Thus a "positive" hippuric acid test, indicates not only that glycine synthesis is disturbed, but signifies that in all probability the functional capacity of the liver as a whole is reduced.

Drill and Ivy (1944) point out that more emphasis should be laid on the "correlation" or "association" of liver functions than on their "dissociation" and this aspect is also borne out by the observation that positive results to the most sensitive tests in a given condition are followed by other tests indicating abnormal function as the degree of damage increases.

Detection of Hepatic Dysfunction as an Aid in Diagnosis : the Lack of Specificity of Liver Function Tests

In speaking of the "diagnostic value" of liver function tests a distinction should be made between cases with jaundice where the involvement of the liver is often obvious, and cases with suspected "latent" liver disease. A test which fails diagnostically in the first group in distinguishing between "surgical" and "medical" jaundice may nevertheless be valuable in the latter group where the demonstration of hepatic damage or dysfunction may support the clinical diagnosis. In using tests for this purpose there must be taken into account the variety of illnesses other than hepatobiliary disease which may be associated with disturbed hepatic function. These include acute and chronic infections such as pneumonia (especially atypical pneumonia), rheumatic fever,

endocarditis, rheumatoid arthritis, diphtheria, malaria, low grade trivial infections of various origin, urinary infections, pulmonary tuberculosis, ulcerative colitis, idiopathic steatorrhoea, malnutrition in general, Addison's disease, pellagra, neoplasm in general and in particular carcinoma of the stomach,¹ peptic ulcer, allergic conditions, certain anaemias, notably pernicious anaemia, congestive heart failure, hyperthyroidism, toxæmic pregnancy, during menstruation, following surgical operations, following arsenical and sulphonamide treatment, etc.

The laboratory is not a slot-machine, nor are liver function tests a short cut to a clinical diagnosis; but if regard is paid to these limitations and the tests are viewed against the clinical background, some will be of diagnostic value where the clinical diagnosis depends in part on the demonstration of hepatic damage in the laboratory. In much the same way as the significance of other non-specific phenomena such as sedimentation rate and white cell changes is in clinical practice evaluated for diagnostic purposes.

Thus any test revealing hepatic damage or dysfunction might be used diagnostically. For example, "the qualitative urobilinogen test (in the urine), especially if used serially and if strongly positive, may be quite sufficient in company with the history and physical findings, to permit the diagnosis of cirrhosis." The same holds for other tests, and every physician interprets in this way changes in serum proteins, which are by no means pathognomonic.

The colloidal gold reaction, the thymol turbidity test, Hanger's cephalin cholesterol flocculation test (1939) and the Takata-Ara reaction deserve special mention. These empirical tests, depending on qualitative changes of the serum proteins (see p. 146) appear to be very sensitive in the detection of active hepatic disease. MacLagan's modification (see p. 140) of the colloidal gold reaction has, in his hands, proved fairly specific and of considerable differential diagnostic value. The fact that positive results are obtained in heart failure, severe chronic anaemia, and in various acute and chronic infections, particularly glandular fever, atypical pneumonia, advanced pulmonary tuberculosis, infective endocarditis, rheumatoid

¹ Evidence of hepatic dysfunction in patients suffering from carcinoma does not necessarily prove the presence of hepatic metastases. The extensive metabolic studies of Rhoads and his associates (Abels *et al.*, 1942, 1943) on patients with cancer of the gastro-intestinal tract, particularly cancer of the stomach, suggest that the very presence of cancer, even when restricted to its site of origin, imposes on the liver functional disturbances which are in part reversible. Our observations with the hippuric acid test in patients with cancer of the stomach confirm this.

arthritis, etc., must be kept in mind, but will not usually seriously upset interpretation.

A high degree of sensitivity has been found with Hanger's flocculation test (Pohle and Stewart, 1941) but its differential diagnostic value in jaundice is not confirmed. The prognostic value of the test is stressed by Hanger and Patek (1941). Although negative tests do not exclude cirrhosis, they indicate a favourable prognosis, and the reverse holds for persistently positive reactions. Dick (1945) in this country has recently reported satisfactory results with this test.

MacLagan (1944a, 1945) has recently described a new test which falls in this group, the "Thymol Turbidity Test"; it is technically very simple, rapid and apparently a very sensitive indicator of liver dysfunction. (See p. 149) The results with this test in hepatitis, cirrhosis and obstructive jaundice (186 cases) have been almost identical with those obtained with the colloidal gold reaction, but the test was less often positive in chronic infections, and therefore probably more specifically related to liver disease, though in the positive reactors with infections corroborative evidence of hepatic dysfunction was obtained with other tests.

The Takata-Ara test still remains of value as presumptive and corroborative evidence of hepatic involvement. Ucko's modification (1942) gave positive results in almost all (99 per cent.) cases of Laennec's atrophic cirrhosis, but it is usually negative in "simple jaundice" and a positive reaction here indicates more serious parenchymatous damage. During the last few years the test has been frequently found positive in cases of "infective hepatitis." The occurrence of positive reactions in other, non-hepatic, diseases is about the same as with the other three tests, and this seems to indicate disturbances of protein metabolism in these conditions for which probably an involvement of the liver is responsible. The test is of prognostic value. Patients with gall-stones should not, except in emergency, be operated upon if their serum reaction is strongly positive.

DIFFERENTIAL DIAGNOSIS OF JAUNDICE

On the whole liver function tests are more useful in judging the degree of damage and surgical risk and in following progress than in aiding differential diagnosis. While at present there is no single test or group of tests which will reliably differentiate in every case, there are a few procedures which do assist in the differential diagnosis.

The reason for this unsatisfactory situation lies rather in the complicated pathology of hepatic disorders than in the inadequacy of the tests. Extra-hepatic obstructive jaundice is usually associated with some parenchymatous damage which tends to increase with the duration of the obstruction, but may be present early, especially in the presence of inflammatory changes of the biliary tract, or with recurrent obstruction. On the other hand, parenchymatous jaundice is probably always associated with some injury or obstruction to the bile ducts, possibly together with suppression of bile secretion, and it is particularly this occurrence of intra-hepatic biliary obstruction in hepatitis which is responsible for one of the chief difficulties in differential diagnosis. The need for considering laboratory findings in conjunction with the clinical information and especially with the course of the illness cannot be over-emphasized, though in a fair proportion of cases a correct diagnosis could be made blindly from a particular combination of the results of several tests.

The crux of the problem is to separate "medical" (hepatocellular) from "surgical" (extra-hepatic obstructive) jaundice, and this is more important than to make a specific diagnosis. A misdiagnosed "medical" jaundice may be a surgical calamity, the surgical mortality being from about 35 per cent. in cirrhosis to nearly 100 per cent. in severe acute hepatitis and yellow atrophy. "Cases of jaundice are rarely emergencies" and a period of study is useful. In cases of prolonged jaundice with continued acholia due to intra-hepatic biliary obstruction, or even with "surgical" obstruction, the liver will in most cases preserve a fair degree of function, or regain it after acute inflammatory processes have subsided; and those patients with severely damaged livers are in need of a period of pre-operative treatment.

In the majority of cases of jaundice the diagnosis can be made from the history and clinical findings, together with the simple qualitative urine tests for urobilinogen and bilirubin. A very helpful review of the differential diagnosis of jaundice is given by White (1943, 1944)

Bile Pigment Metabolism

Urobilinogen Excretion: Bilirubin is formed from haemoglobin by the reticuloendothelial system and circulates in the blood in a protein-bound form which does not pass through the kidneys. It is taken up by the liver and excreted with the bile into the

intestine where it is reduced by bacteria to urobilinogen (stercobilin). Most of it is excreted with the faeces (40-280 mg. per day) but some is reabsorbed and reaches the liver via the portal blood stream, and is re-oxidised there to bilirubin. A part of the urobilinogen by-passes the liver and appears in the urine (normally not more than 3 mg. per day). Damage to, or increased strain on, the liver impairs its capacity to reconvert the urobilinogen (or urobilin) to bilirubin, and the resulting increase in urinary urobilinogen (UU), demonstrable in fresh urine by the simple qualitative Ehrlich's test, is still one of the most sensitive signs of impaired liver function. In complete biliary obstruction with no bilirubin entering the intestine, no urobilin is formed and none appears in the urine. In this case the urine does not, as it normally does, develop a pink colour on heating with the Ehrlich aldehyde reagent.

The simple qualitative (or semi-quantitative) test for urobilinogen in the urine, serially performed is the most important single procedure for the differentiation of jaundice. Additional information of value is obtained from repeated determinations of bilirubin in the serum, and serial quantitative determination of urobilinogen in the faeces, the latter being of special importance for the diagnosis of complete obstruction.

The quantitative data obtained by Watson (1937, 1940) are used below to serve as a guide to the interpretation of results obtained with simpler tests. The technique of urobilinogen tests is given on p. 154.

Jaundice due to neoplasm is in 90 per cent. of cases complete, and rarely improves, and the amount of faecal urobilinogen (FU) will usually be less than 5 mg. per day, the urine showing no urobilinogen or traces only.

Jaundice due to stones in the biliary tracts is rarely associated with complete obstruction and will seldom show persistent values of FU below 5 mg. The jaundice characteristically fluctuates. Urinary urobilinogen (UU) may be absent or slightly increased (0-50 mg) except in the presence of complicating factors, such as acute cholecystitis, cholangitis or cirrhosis, when it may be considerably raised.

In parenchymatous jaundice (infective and toxic hepatitis, sub-acute hepatic necrosis, cirrhosis) it is common to find a marked increase in UU (up to 1,000, usually 10-300 mg. per day) the FU usually giving low normal figures except in cases with an additional component of blood destruction. Thus a typical finding is an

inversion of the UU/FU ratio, indicating severe liver damage, though the UU may be completely absent in spite of the presence of FU ranging up to 10-20 mg. per day, indicating that the liver is still capable of disposing of the small amount of absorbed urobilinogen. A phase characterized by acholic stools and absence of UU and very low FU, exhibiting the picture of complete biliary obstruction is not uncommon, particularly in toxic hepatitis. In infective hepatitis it usually lasts only a few days and rarely longer than two weeks, but it may extend over several weeks in subacute necrosis of the liver. Watson points out that here the grossly acholic stools may still contain from 10 to 15 mg. of urobilinogen per day, and a 24-hour specimen of urine giving a negative qualitative Ehrlich's test may yield as much as 15 mg.

Serial urine tests for urobilinogen and bilirubin will show a characteristic pattern in cases of *parenchymatous jaundice with intra-hepatic obstruction*. The urobilinogen in the urine at first increases, then during the phase of bile suppression diminishes or disappears from the urine, but rises again for several days during recovery (re-establishment of bile flow) before eventually dropping to normal values; the bilirubin excretion is greatest during the obstruction phase. The increase in urinary urobilinogen excretion is characteristic of intra-hepatic obstruction and is usually not marked or is absent after release of obstructive jaundice of not too long duration due to mechanical extra-hepatic causes.

Thus, while *absence* of urobilinogen from the urine occurs in partial obstruction due to stone and also in parenchymatous jaundice, though in the latter rarely for more than 4-7 consecutive days, *presence* of urobilinogen in the urine is strong evidence against neoplastic biliary obstruction.

Uncomplicated Haemolytic Jaundice (acholuric jaundice) is readily identified by the presence of a strongly positive indirect van den Bergh reaction (*vide infra*) with a negative direct, together with the characteristic haematological appearances of anaemia, reticulocytosis, spherocytosis and increased erythrocyte fragility, together with increase of the urobilinogen in urine and faeces. The UU is usually only slightly raised (1 to 200, average 5 to 30 mg.) but that of the FU enormously so (300 to 4,000, usually 600 to 2,000 mg.).

Serum Bilirubin (Van den Bergh Reaction): This test is of direct diagnostic value in uncomplicated haemolytic jaundice where it gives an "indirect" reaction. When it is certain that the "jaundice" is due to hyperbilirubinaemia, the simpler icterus index may be used.

Other tests of help in the differential diagnosis of jaundice are the colloidal gold reaction and the thymol turbidity test of MacLagan, especially in conjunction with the serum alkaline phosphatase and also the galactose tests and the plasma prothrombin level and its response to vitamin K. They will be discussed in detail here in connection with the differential diagnosis of jaundice, to avoid repetition later, though some of them are probably more useful in other spheres.

Some of these tests, if not of diagnostic help may in many cases aid in prognosis, in assessing surgical risk, and in indicating the need for and the efficacy of treatment.

The Serum Colloidal Gold Reaction

This test is considered a most sensitive indicator of active hepatic disease but does not measure any known function. The reaction apparently depends on qualitative changes within the globulin fraction of the serum (excess of gamma globulin) and not on an inversion of the albumin/globulin ratio.

Gray's test (1940) gave positive results in all cases of hepatic cirrhosis, and in a high proportion of other cases with generalized liver damage, it was also frequently positive in obstructive jaundice (Noth and Loew, 1943).

MacLagan's modification (MacLagan 1944, 1944b) (see p. 147) appears to have a selective sensitivity different from that of Gray's original test, rendering it more suitable for the differentiation of mechanical obstructive from parenchymatous jaundice. Normal subjects give uniformly negative results. The results in 319 cases show that the test is usually positive in infective hepatitis (93 per cent.) and in cirrhosis, but less (44 per cent.) frequently positive in arsenical jaundice; it is negative in jaundice due to extra-hepatic obstruction (93 per cent.) and usually remains negative during the first 3 months in spite of increasing liver damage as shown by other tests; it is therefore of value in distinguishing this condition from the first two. A strongly positive gold reaction (4 or 5 plus) has not been seen in obstructive jaundice, and is thus of more help in this connection than a negative result which does not absolutely exclude infective hepatitis. The diagnostic value is considerably increased if used together with the phosphatase test. To summarize, three particular combinations have diagnostic significance:

(A) A negative gold reaction with phosphatase above 35 King-Armstrong units suggests biliary obstruction.

(B) A positive gold reaction with phosphatase below 25 suggests parenchymatous hepatic disease and absence of biliary obstruction.

(C) A strongly positive gold reaction (4 or 5) has not been seen in obstructive jaundice and appears to be diagnostic of parenchymatous disease at any phosphatase level. ✓

The following table includes all jaundiced patients on whom both tests were done. It will be seen that out of those 100 cases, 75 fall into group A, B or C (Columns 1, 3 and 4) and in all of these the findings were in keeping with the final diagnosis

COMBINATION OF TWO TESTS IN 100 JAUNDICED PATIENTS.

	1	2	3	4	
Gold reaction Phosphatase .	Negative Over 35	Negative Under 35	Positive Over 25	Positive Under 25	Total
Obstructive Jaundice ¹ .	22	9	1 (1+)	0	32
Infective hepatitis .	0	7	1 (5+)	28	36
Cirrhosis and subacute hepatitis .	0	0	4 (5+)	6	10
Arsenical Jaundice .	0	6	1 (5+)	12	19
Toxic Jaundice .	0	2	0	1	3
TOTALS . .	22	24	7	47	100

¹ Due to pancreatic carcinoma in 14, gall-stones in 9, hepatic metastases in 6 and other causes in 3.

Figures in brackets indicate strength of gold reaction.

The results given by MacLagan indicate that "out of 100 jaundiced patients the distinction between biliary obstruction and generalized liver-disease could be made in 75 cases with gold and phosphatase tests, and in still higher proportion if the galactose index was included" Results with the thymol turbidity test have been very similar to those with the colloidal gold reaction.

In conclusion, MacLagan's modification of the colloidal gold reaction and also the thymol turbidity test appear two of the most promising tests for giving assistance in the differentiation of jaundice.

Serum Alkaline Phosphatase

The estimation of blood phosphatase as a diagnostic test in the differentiation of jaundice was first suggested by Roberts (1933).

A rise of "alkaline" phosphatase in the blood results from interference with the outflow of bile and occurs also in various types of hepatic damage,¹ where it is associated with an increase in the concentration of phosphatase in the bile.

The rise in serum alkaline phosphatase is on the whole more marked in jaundice due to extra-hepatic obstruction than in parenchymatous jaundice. In severe jaundice due to gross mechanical obstruction the phosphatase level is always above normal and usually markedly raised; in the "hepatitis" group it is in many cases normal, in others more or less raised. Although this overlapping of values compromises the absolute diagnostic value of the test it gives aid in clinical differentiation. Thus marked jaundice in a patient with normal or slightly raised phosphatase is unlikely to be due to extra-hepatic obstruction. On the other hand, a high phosphatase level (above 35 units) together with a normal colloidal gold or thymol reaction indicates "surgical" jaundice (see under Colloidal Gold Reaction, p. 140).

In the absence of jaundice and other limiting factors, an elevation in the serum alkaline phosphatase may be a sensitive indicator of liver damage.

The co-existence of bone disease in which the alkaline phosphatase may be increased (including Paget's disease, hyperparathyroidism, benign and malignant tumours, bone metastases, rickets and tuberculosis should be excluded.

Galactose Tolerance Test

Since the work of Bauer in 1906, the ability of the liver to assimilate galactose has been used to test hepatic function and for the differentiation of jaundice.

Oral Test (Bauer) Excretion of over 3 g of galactose in the urine over a period of 5 hours after oral administration of 40 g. indicates hepatic impairment

Bensley (1935) reviewing the results of previous investigators and his own, found the test to be positive in about 70 per cent. of cases of parenchymatous jaundice, and negative in about 80 per cent. of obstructive jaundice. It is important to do the test early within the first two weeks of the jaundice, as later on parenchymatous damage may supervene in extra-hepatic obstructive jaundice.

¹ Although the degree of injury to hepatic cells appears to be one of the factors determining the accumulation of the enzyme in the blood, it is not certain to what extent a release of the enzyme from liver cells or other tissues, or possibly interference with its excretion by the liver is involved

The following two modifications are considered more sensitive :

Oral Test with Estimation of Blood Galactose (MacLagan, 1940 ; MacLagan and Rundle, 1940) : After oral administration of 40 g. of galactose the blood galactose is estimated at intervals of $\frac{1}{2}$, 1, $1\frac{1}{2}$ and 2 hours. In normals the peak of the curve usually occurs at one hour and does not exceed 80 mg. per cent. ; the sum of the four half-hourly values (galactose index) should not exceed 160, and averages 68 mg. per cent. The test is usually normal in obstruction of short duration but may become positive after prolonged, or repeated attacks of, jaundice. It is nearly always positive in hepato-cellular jaundice.

Intravenous Galactose Test (Bassett, Althausen and Coltrin, 1941 ; King and Aitken, 1940). The blood galactose is estimated 75 minutes after intravenous injection of 1 ml. of 50 per cent. galactose solution per kg. body weight ; by this time the blood is normally cleared of galactose. In 80 per cent. of cases of obstructive jaundice of shorter duration than 6 months the galactose retention at 75 minutes was below 20 mg. per cent. while it was above that level in 81 per cent. of cases of hepatitis and in 97 per cent. of hepatic cirrhosis. The test is of value in distinguishing parenchymatous from obstructive jaundice.

The discrepancies in the results obtained with the last two tests in hyperthyroidism have been discussed by Barnes and King (1943).

Plasma Prothrombin

A measure of the prothrombin level is in the jaundiced patient believed to be of help (a) in controlling the bleeding tendency ; (b) in the diagnosis of the type of jaundice, and (c) in gauging the degree of hepatic damage.

The liver is of importance in forming prothrombin from vitamin K and, provided a normal supply of vitamin K from the intestine, the prothrombin level represents an index of hepatic function. A low prothrombin level in the jaundiced patient may be due to hepatic damage and/or malabsorption of vitamin K resulting from biliary obstruction, and the response to administration of vitamin K

of severe hepatic damage and suggests that the hypoprothrombinaemia was due mainly to lack of vitamin K secondary to the

biliary obstruction. A failure to respond to vitamin K, commonly observed in hepatitis and cirrhosis, indicates hepatic damage, either primary or secondary to the biliary obstruction. In the latter case it is of use in indicating employment of therapeutic measures directed at improving hepatic function before surgical intervention.

Interpretation is limited by the observation that the plasma prothrombin level is not always subnormal in parenchymatous jaundice and that, on the other hand, the hypoprothrombinaemia with healing parenchymatous jaundice may respond to vitamin K therapy similarly to extra-hepatic biliary obstruction (Lichtman, 1942; White, Deutsch *et al.*, 1942).

Summary

In the differential diagnosis of jaundice isolated laboratory findings are of little use and usually not conclusive without clinical data. The emphasis is on simple tests, serially repeated. Of these the most valuable are serial qualitative urobilinogen tests on the urine, quantitative urobilinogen estimation on the faeces, MacLagan's modification of the colloidal gold reaction, the thymol turbidity test, especially together with the alkaline phosphatase test, and galactose tolerance tests; important additional information is provided by plasma prothrombin time and its response to vitamin K.

ESTIMATION OF THE EXTENT OF HEPATIC INSUFFICIENCY

The value of certain liver function tests in indicating the need for treatment and assessing progress and prognosis is increasing with the development of therapeutic measures; in particular they afford aid in the pre- and post-operative management of surgical patients. The depression of liver function which follows, in particular, abdominal surgery is more serious in patients with damaged livers and in these the mortality is higher and the post-operative course less favourable. The detection of impaired liver function, which may be clinically unsuspected, is of help in assessing surgical risk and in identifying cases demanding special pre-operative measures. Serial tests are particularly valuable. The following are suitable for this purpose.

The Bromsulphalein Clearance Test

This test is based on the assumption that the dye is removed from the blood mainly by the liver. It consists of the intravenous injection of 5 mg of the dye per kg. of body weight. Blood samples are withdrawn at 5 and 45 minutes after the injection, and the percentage retention of the dye at 45 minutes is estimated by comparison with prepared standards. In normals the blood is clear of the dye within 45 minutes.

The test is one of the most sensitive and reliable measures of hepatic insufficiency, in the absence of jaundice, and of great prognostic value, especially in surgical patients.

THE PROTHROMBIN TEST has been discussed. Operative risk and prognosis are bad if administration of vitamin K fails to restore the prothrombin time to normal, and Ivy and Roth (1943a) believe it to be the most valuable single test.

The Hippuric Acid Test

The basis of the test is the synthesis of hippuric acid (HA) from benzoic acid, administered as sodium benzoate, according to the equation $\text{Benzoic acid} + \text{glycine} = \text{hippuric acid} + \text{water}$. This synthesis involves two mechanisms the nature of which is not yet fully clarified (Borsook and Dubnoff, 1940), but which appear to be controlled by the liver: First, the production of glycine which is believed to be formed by the liver in response to the benzoic acid administered, and then the conjugation of glycine with benzoic acid to form HA. The reduced output of HA in patients with liver disease is probably due mainly to reduced capacity of the liver to furnish glycine (Probstein and Londe, 1942) and in part due to a disturbance in the conjugation. The latter process has in man not actually been proven to take place in the liver, but Snapper and Gruenbaum (1924) have demonstrated that the human kidney takes part in the conjugation, apart from influencing excretion of formed HA. Damage to the liver may, therefore, be thought to interfere with the synthesis of HA in part indirectly, by affecting the kidneys and this possibility is supported by evidence of a hepato-renal interrelationship, *i.e.* of functional and anatomical changes of the kidneys associated with, and in part probably secondary to, liver damage with and without jaundice (Meyer *et al.*, 1941). It seems difficult in some cases to draw the line between primary kidney damage and renal disturbances secondary to or aggravated by liver

damage; it is believed by some that in presence of a normal blood urea the kidneys may be trusted to play their part (Londe and Probst, 1941) while others (Hirsch, 1935; Kohlstaedt and Helmer, 1936) have attempted to control the renal factor by means of the urea clearance test. This theoretical complication may explain the fact that the output of HA is frequently considerably reduced in mechanical obstructive jaundice when other tests fail to show hepatic damage and renders the test of limited value. Such considerations are less serious if the test is employed as a measure of hepatic insufficiency, particularly in follow-up studies. Apart from renal failure and liver disease, low results have been found in anaemia and other conditions by Sherlock (1946).

In planning partial gastrectomy for simple peptic ulcer we prefer to wait until the excretion of HA (oral test; 6 g. dose) is at least over 3 g., preferably over 4 g.

(For technique, see Appendix.)

Serum Proteins

Numerous clinical studies on changes in serum protein in hepatic disease (Higgins *et al*, 1944; Post and Patek, 1942, 1943) together with experimental evidence (Madden and Whipple, 1940; Loeb, 1941) show the importance of the liver in the regulation of plasma proteins.

The normal range for serum albumin is from 4-5.5 g. per cent. and for globulin from 1.4-3.0 g. The serum albumin tends to be moderately reduced in *acute* hepatitis but may be greatly so in severe and protracted (subacute and chronic) cases; but low albumin values are usually associated with a rise in globulin. A qualitative change in the globulin component, apparently residing in the euglobulin fraction (Gray and Barron, 1943; MacLagan, 1945) is present and seems to be characteristic of liver damage; it is reflected by flocculation tests such as the colloidal gold, the cephalin flocculation, the Takata-Ara and thymol turbidity reactions.

The tendency toward reduction of serum albumin and elevation of globulin is more marked in *chronic* liver disease, notably in cirrhosis of the liver; the changes in albumin levels are of prognostic significance, the outlook being poor below 2.5, favourable in cases where it rises to normal levels and remains there, and doubtful where it does not reach normal levels or improves only temporarily (Post and Patek, 1943).

APPENDIX: METHODS

The Serum Colloidal Gold Reaction

MacLagan (1944) found that the precipitation of the gold sol was greatly influenced by the reaction and the ionic strength of the medium. The technique of Gray was modified by introduction of a buffer solution, which permits the test to be performed in a single tube in a reproducible manner without preliminary standardization of the gold sol. The conditions chosen give a final serum dilution of 1 in 60, a pH of 7.8, and an ionic strength of 0.01.

Solutions Required

A. The Colloidal Gold Sol: The use of a gold sol of a fairly constant particle size is essential and other sols, *e.g.* those prepared by the citrate method are much less sensitive and therefore unsuitable. An essential part in preparing the gold sol consists in the "preliminary titration," on the basis of which the gold sol is then prepared in bulk. The following technique is recommended by MacLagan; it has been used for the last three years without failures and produces a stable sol which is equally suitable for the use with serum or cerebrospinal fluid.

The whole method is designed to produce a fine adjustment of pH without buffers, a simple but somewhat delicate method. For this reason the glassware must be chemically clean, and is rinsed with double distilled water just before use. The heating rate is of paramount importance.

Reagents

- (1) *Double distilled water* · Ordinary distilled water redistilled from glass. It is desirable to prepare an adequate bulk of this before commencing operations.
- (2) *Gold chloride solution* · 1 g. $\text{AuCl}_3 \cdot \text{NaCl} \cdot 2\text{H}_2\text{O}$ in 100 ml. double distilled water.
- (3) *Potassium oxalate solution*: 1 g. of AnaR reagent $(\text{COOK})_2 \cdot \text{H}_2\text{O}$ in 100 ml. double distilled water.
- (4) 0.02 N. *Sodium hydroxide*.

Preliminary Titration: Add to 50 ml. of double distilled water in a 100 ml. conical flask, 0.5 ml. of gold chloride, 0.5 ml. of potassium oxalate, and (say) 1.5 ml. of NaOH. Heat at once with a flame so adjusted that the mixture reaches the boiling point in 2½ to 3 minutes. If the result is successful the mixture will turn from bluish red to dark red at or near the boiling point and will then suddenly lighten in colour. If these changes do not occur after 2 minutes boiling, repeat the procedure with a different amount of alkali. As a guide to this titration

too much alkali produces a pale or purple sol, and too little a turbid or "muddy" sol. The end point is sensitive to about 0.1 ml. so that if, e.g. 1.6 ml. of NaOH is the optimum, the 1.5 or 1.7 ml. are not so good. The correct sol is clear red by transmitted light but has a definite sheen when viewed by reflected light.

Preparation of Bulk Lots: Having determined in this way the amount of alkali required for the particular batch of reagents in use, prepare bulk lots with ten times the above quantities, using 1 litre conical flasks and a source of heat sufficient to reach boiling point in $5\frac{1}{2}$ to 6 minutes. Three Bunsen burners are usually adequate and two of these are turned out when the mixture boils. Reject any batch which does not exhibit the sequence of colour changes described above. A further slight adjustment in the amount of alkali is occasionally needed. Finally pool all successful 500 ml. lots.

Management of the Sol: The sol should be stored in glass-stoppered Winchester quart bottles which have previously contained strong acids, with some form of dust cover. The sol is never pipetted direct from the bottle nor are any unused portions returned to the main bulk. Under these conditions it will keep without significant change for at least six months. For the serum colloidal sols reaction it is not necessary to standardize the sol in any way as would be done for the Lange CSF test as the final reaction is controlled by the buffer solution.

B. Barbitone Buffer (pH 7.8, 0.02 M): 0.552 g. of barbitone (di-ethylbarbituric acid), 0.412 g. of sodium barbitone, and 0.2 g. of phenol are dissolved in 100 ml. of water with the aid of heat. This solution keeps for at least 2-3 months, the phenol serving to prevent the growth of moulds.

Method

Measure 0.05 ml. of serum into a dry test tube 4 in. by $\frac{1}{2}$ in., using a 0.2 ml. pipette with a waxed tip. Add 0.5 ml. of buffer and then 2.5 ml. of gold sol. The final ionic strength is about 0.01 including serum electrolytes and gold sol. Shake and allow to stand overnight. There is no change in tint as in the Lange test, but positive results are usually indicated by a turbidity appearing within 5-10 minutes. Record results as 5 complete precipitation, 4 supernatant fluid just coloured, 1 gold precipitate just visible on shaking, 2 and 3 being intermediate grades of precipitation, judged mainly by the colour of the supernatant fluid.

The test can be made semi-quantitative by adding 0.1 ml. of serum to 0.9 ml. of buffer, mixing and transferring 0.5 ml. to the tube containing 0.5 ml. of buffer and so on. Six tubes are usually

sufficient, complete precipitation being rare after the fourth tube (0.00625 ml. of serum).

Typical results in following up at weekly intervals the course of infective hepatitis :

555400 552000 530000 300000 000000

Serum for the test was found to keep for several weeks in the refrigerator without change and slight degrees of haemolysis do not interfere. Heating to 56° for 30 minutes partly abolishes the reaction, so that inactivated sera cannot be used.

The Thymol Turbidity Test

(MacLagan, 1944, 1945)

The test appears to depend on an increase in serum gamma globulin and seems chemically related to the Pandy and the cephalin-cholesterol test. Changes in pH and ionic strength exert a marked influence on the precipitation. The test is outstanding by its simplicity which is combined with a high degree of sensitivity. The results run closely parallel to those with the colloidal gold test except in Weil's disease (more often positive) and in chronic infections (less often positive).

Reagents

Thymol Buffer, pH 7.8, 0.01 M: Add 500 ml of distilled water to 1.38 g. of barbitone, 1.03 g. of sodium barbitone, and approximately 3 g. of thymol. Heat just to boiling point, shake well, and cool thoroughly. The mixture should now be turbid. Seed with a small amount of powdered thymol crystals, shake, and allow to stand overnight at a temperature of 20–25° C. Finally shake again well (to avoid super-saturation) and filter the clear solution from the crystalline deposit.

(It is not possible to shorten this technique by weighing out an exact amount of thymol, because there is some interaction between the thymol and barbitone on heating, presumably esterification, and an excess of thymol must be present to ensure saturation. The solubility of thymol is significantly reduced below 20° C.)

Method

Measure 1 volume of serum (0.05 ml. from a 0.2 ml. graduated pipette with a waxed tip) into a dry tube and add 60 volumes of buffer (3 ml.). Allow to stand for half to one hour and compare in a comparator with a black line on a white background against

the turbidity standards used for urine protein estimation¹. When the result is positive, flocculation frequently occurs on standing overnight but this is not an essential part of the test. If the turbidity exceeds the 100 mg. per cent. standard, dilute with a further measured volume of buffer as required. The result is expressed in arbitrary units equal to the appropriate standard divided by ten with allowance for dilution. The standard dilution is 1 to 60 so that if e.g. the final dilution is 1 to 120 and the mixture then matches the 70 mg. per cent. tube, the result is 14 units.

$$\text{i.e. Units} = \frac{\text{Standard tube reading} \times \text{Final dilution of serum}}{600}$$

Normal limits are 0 to 4 units. The reaction is partly abolished by heating to 56° C. for 30 minutes, so that inactivated sera cannot be used. The standards should be checked every few months against diluted serum of known protein content, using one volume of diluted serum to 3 volumes of 3 per cent. sulphosalicylic acid.

The Takata-Ara Reaction ✓

(Ucko's modification, 1936, 1942)

The reaction consists in a precipitation of serum protein by addition of sodium carbonate and mercuric chloride and it is probably dependent chiefly on qualitative changes in the serum proteins.

This modification is simpler than the original Takata reaction giving a clear result after only ninety minutes (as compared with 24 hours with the original method) and allowing a gradation of the results from + to +++.

Reagents

- (1) 0.36 per cent. solution of *anhydrous* sodium carbonate.
- (2) 0.5 per cent. solution of mercuric chloride (sublimite).

Method

Into 5 clean test tubes of 11 mm. diameter, each containing 0.2 ml. of serum, are measured 0.1, 0.15, 0.2, 0.25 and 0.3 ml. of the sodium carbonate solution. After shaking, the same quantities of the sublimite solution are added and the tubes are shaken again

¹ I.e. 1 ml. of solutions of various protein contents + 3 ml. salicylsulphonic acid

and allowed to settle. Readings by daylight are taken at once and after 90 minutes.

- (1) Reaction +++ . A thick precipitation in all the tubes forms *immediately* after addition of the reagents.
- (2) Reaction ++ . All tubes show a uniform turbidity and are not translucent.
- (3) Reaction + . The first three tubes are turbid and not translucent, the remaining two being translucent.
- (4) Reaction negative . Three or more tubes are translucent. The mixture may be clear or show a slight opacity.

The solutions are stable for a month if kept in the dark and at 0° C. They should be checked from time to time by adding a drop of phenolphthalein to a mixture of equal parts of the two solutions. If accurate, the mixture shows a weak pink colour.

The Hippuric Acid Test (Quick)

Determination of Hippuric Acid in the Urine: Weichselbaum and Probst (1939) found that Quick's simple precipitation method (1933, "No. 1") gave only partial recovery of hippuric acid (HA) from the urine, and that addition of 30 per cent. of NaCl to the urine appreciably decreased and stabilized the solubility of HA. This has been confirmed by others and by Quick himself who recommended addition of 50 per cent. of ammonium sulphate. Comparison of the two methods shows an increase in the yield of HA by the NaCl modification up to 33 per cent. (Hepler and Gurley 1942; Marron, 1941).

It is recommended to carry out in detail the technique as described by Londe and Probst (1941), except that an excess of conc HCl was found an advantage.

Oral Test Procedure

On the morning of the test, one hour after a light breakfast of weak tea or coffee and bread and butter, give the patient the test dose of sodium-benzoate (4 or 6 g.) dissolved in 30 ml of water, preferably flavoured with oil of peppermint. Follow with half a glass of water from the same glass. The patient had previously emptied his bladder and this urine is discarded. All urine passed for the next 4 hours is collected for examination and the volume measured.

If the HA is determined in a pooled 4 hour specimen, no reduction of the volume is necessary if it is below 600 ml. Add a few drops of glacial acetic acid. The volume is reduced in a water bath if greater than 600 ml. or if the amount of HA is expected to be very small, or alternatively, a known amount of HA may be added. Then add 30 g. of NaCl per 100 ml. of urine, and heat until the salt is dissolved. Cool the solution to 15° or 20° C by immersion of flask into ice-cold water. Add 2 ml (or more) of conc. HCl and scratch vigorously the inside of the flask with a glass rod until precipitation of the HA occurs. (This step is important) Allow for 15 minutes to stand in the cold water bath and then filter through a Hirsch funnel (diameter of perforated plate, 47 mm), using moderate suction. Wash the precipitate with cold 30 per cent NaCl solution from a wash bottle, using the washing fluid first to rinse the flask in which the precipitation has been performed. The precipitate is adequately washed when the washing fluid is free of HCl (titrate with NaOH and phenolphthalein). Transfer the funnel with its contents into the flask that still contains some of the HA crystals, and rinse the filtered HIA into it by dissolving it in hot water, using a fine tipped wash bottle. All of the HA is now in the flask in which it was precipitated. Heat until all the HA particles adhering to the flask dissolve, and titrate while hot with 0.5 N. NaOH with phenolphthalein as an indicator.

The number of ml. of 0.5 N NaOH used for neutralization is multiplied by 0.072, which gives the amount of HIA precipitated in terms of sodium benzoate. To this amount add the correction for the solubility of HA (in terms of sodium benzoate), i.e. 0.10 g. of sodium benzoate for each 100 ml. of urine. The result is the HA of the specimen expressed in terms of sodium benzoate with a maximum error of 10.5 mg. per 100 ml. Bile or protein in the urine interferes with the procedure but can be removed by the following method (M. Somogyi): Add 5 ml. of 30 per cent zinc sulphate and then 2.5 ml. of 2.5 N-NaOH per 100 ml of urine, shake and filter. With large quantities of protein heat to near boiling point on steam bath before filtering. For the rest the procedure is the same except that allowance must be made for dilution with the precipitants.

Lichtman (1942) recommends the following procedure for markedly bile-stained or dark-coloured specimens of urine: Add 0.3 g. of acid washed norit per 100 ml. of urine and boil the mixture for about 1 minute. After cooling filter by suction on a Hirsch funnel, and wash the norit residue with a small quantity of hot water. Measure the volume of the filtrate and proceed as outlined above.

Interpretation

The normal figures obtained with the analytical method described here are *higher* than those reported by Quick and others

using the original precipitation method. This is probably in the first place due to the improved method, and secondly due to the selection of normal controls which, as the literature reveals, often were recruited from hospital patients free from obvious liver disease but suffering from conditions associated with impaired liver function.

Using a test dose of 6 g. of sodium benzoate we found the excretion of HA (in terms of sodium benzoate) in 4 hours, in a group of 30 perfectly healthy male subjects between the age of 20 and 60 years, to range between 4.0 and 5.88 g., with a mean of 4.90 and a standard deviation of 0.34 g. These figures are practically identical with those obtained by Probstein and Londe (1940) and Hepler and Gurley (1942). The latter workers and ourselves found somewhat lower values in women, the range being between 3.36 and 5.49 g. This difference in the two sex groups may be due to a difference in body size which has been shown to influence the excretion of HA, and a formula has been proposed for the predicted normal excretion of HA (for the intravenous test) based on body weight and on surface area (Scurry and Field, 1943).

Probstein and Londe (1940) recommended a test dose of 4 g. of sodium benzoate in preference to Quick's 6 g., as this dose is as fully capable of gauging the maximum rate of HA synthesis as the 6 g. dose, but causing less gastric discomfort. In 24 normal subjects the amount of HA excreted in 4 hours corresponded to between 80 and 90 per cent. of the sodium benzoate ingested; the Oxford group of workers, apparently using the same technique, found in 100 normal controls (50 males and 50 females between the ages of 18 and 50 years) a mean value of 88 per cent. with a standard error of the means of 6.1 per cent.

The Intravenous Test

(Quick, 1939)

An hour or so after a light breakfast have the patient empty his bladder. Then inject slowly, *i.e.* over 5 minutes, a sterile solution of 1.77 g. of sodium benzoate in 20 ml. of distilled water. The patient empties his bladder again 60 minutes after completion of the injection or, as a uniform rate of injection will not always be maintained, 65 minutes from the beginning of the injection.

The urine is carefully measured and the HA determined as outlined before. We prefer to give 200 ml. of water before or immediately after the injection in order to secure an adequate volume of urine. The urine volume should be reduced, after adding a few drops of glacial

acetic acid, in a water bath, if it is over 150 ml or one gram of HA may be added per 150 ml. and dissolved. The same is advisable if the amount of HA excreted is expected to be very small.

The range in normal subjects (expressed in g. of sodium benzoate) is 1.38-0.88 in males, and 1.19-0.71 in females (using the figures of the normal subjects of Scurry and Field's (1943) group).

The intravenous test has the theoretical advantage of eliminating the question of malabsorption, and is also considered to be more sensitive.

The tendency of low urine volumes being associated with low excretion of HA (Pollak, 1943) does not necessarily prove that the former directly causes the latter; both are presumably related to the common underlying disorder, since patients with hepatic disturbances have a tendency to delayed excretion of water and oliguria (Adlersberg and Fox, 1943). Nevertheless, a specimen of urine measuring less than 80 ml. should be regarded with suspicion, partly because with so small a volume the possible error due to incomplete emptying of the bladder may be relatively large.

In over 1,000 tests (half of them intravenous) carried out at the Central Middlesex County Hospital we have had no serious untoward effects. Side effects are minimized if the subject is lying down or sitting after the administration of the sodium benzoate.

Urobilinogen in the Urine

(Ehrlich's Aldehyde Reaction; Neubauer, 1903)

An increase in urinary urobilinogen indicates unpaired liver function provided the amount of urobilinogen absorbed from the intestine is within normal limits. Its absence from the urine for more than 5-7 days usually indicates complete biliary obstruction, but urobilinogen may be absent, usually only for a few days, in partial obstruction and in the "obstructive phase" of hepatitis. Renal damage interferes with its excretion. Its absence in congestive heart failure (where it is, as a rule, increased in the urine) may be taken as indicating renal damage, a finding which should caution against the use of mercury diuretics. The peak of the daily urinary excretion of urobilinogen tends to be reached in the late afternoon. The urine used for the following tests must be fresh.

Methods

To fresh urine is added 1/10 of its volume of Ehrlich's reagent (2 per cent. solution of pure para-dimethylaminobenzaldehyde in 20 per cent.

HCl ¹), allowing 3-5 minutes for development of the colour. Normal urine shows a distinct but faint reddish tinge, which is much intensified by heating, normal urine shows no red tinge in the cold in dilution higher than 1 to 10. The use of the terms "negative" and "positive" test is confusing.

For *practical clinical purposes* it is usually sufficient to know whether urobilinogen is *absent* from the urine (no red colour develops even on heating), present in *normal* amounts (faint pink in the cold), or present in *increased* amounts (distinct red colour in cold urine). In the latter case the degree of the increase may be judged by serial dilution.

Instead of diluting the urine first, the reagent is first added to the undiluted urine, waiting for the maximum colour development. Serial dilutions are made with water until just the faintest pink is discernible, adding 1 ml of the coloured urine specimen to 10, 20, 30, 40, 50, 100 ml. of water and so on.

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¹ Watson (1944) believes that the Ehrlich reagent as described here and widely used is too concentrated. He prefers the following solution. Pure para-dimethylaminobenzaldehyde 0.7 g., concentrated (37-38 per cent) HCl 150 ml., distilled water 100 ml. He recommends the use of sodium acetate in bringing out the maximum colour and in reducing that due to indol or scatol. The test is carried out by adding 5 ml of the Ehrlich reagent to 5 ml. of urine, followed by 10 ml of a saturated aqueous sodium acetate solution.

plastic activity it is necessary to standardize each one against normal plasma (*q.v.*); this is only a minor drawback since the solution of thromboplastin retains its activity unchanged for long periods if stored in the refrigerator in a well stoppered container (Page *et al.*, 1943). The thromboplastin solution need not be standardized at exactly 25 seconds; a correction factor can be applied if the normal clotting time value does not exceed 25 ± 5 seconds.

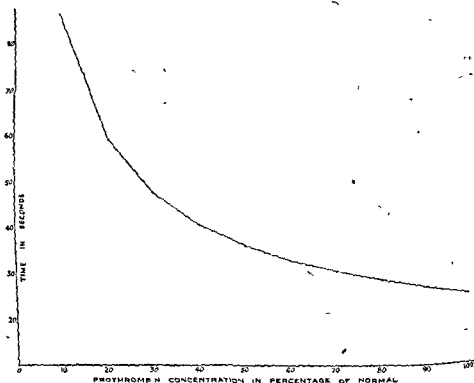


FIG 5.—Curve showing relation between prothrombin concentration and prothrombin time in seconds (Normal clotting time adjusted to 25 seconds)

Technique

Reagents

- (1) Sodium oxalate M/10 (pure anhydrous salt, 1.34 per cent.).
- (2) Calcium chloride M/40 (pure anhydrous salt, 1.11 per cent.).
- (3) Thromboplastin (Russell viper venom, Stypven).

Preparation of Thromboplastin Solution: Dilute the snake venom with distilled water to 1 in 10,000. Test this solution against freshly drawn normal plasma by the technique described below. If the clotting time obtained is below 20 seconds, further dilute the snake

venom solution and test again. By trial and error a solution will be found which gives with normal plasma a clotting time of about 25 seconds. For most batches of venom the appropriate dilution is in the neighbourhood of 1 : 30,000. Store in the refrigerator.

The Test: Blood is drawn by venepuncture and mixed with the oxalate solution in the proportion of 9 parts of blood to 1 part of oxalate. Centrifuge and separate the plasma.

Mix in a small test tube (approx. 6×1 cm) 0.1 ml of plasma and 0.1 ml of thromboplastin solution. Place in the water bath at 37°C . for 1-2 minutes, then add rapidly 0.1 ml. of calcium chloride solution previously warmed to 37°C ., at the same time starting a stop-watch. Mix well for 4-5 seconds; then place again in the water-bath and gently invert the test tube until the first strands of fibrin appear; this is taken as the clotting time. Repeat the estimation 3 to 4 times and take the mean value of the observations.

All glass-ware should be dry and chemically clean.

When the thromboplastin solution gives with normal plasma a clotting time other than 25 seconds the following correction factor is applied:

$$\text{Corrected clotting time} = \frac{25 \times U}{N}$$

where U is the unknown plasma clotting time and N that of the normal plasma.

The clotting time in seconds can be expressed as per cent. of normal prothrombin concentration by referring to the curve in Fig. 1, in which the prothrombin concentrations are plotted against the respective clotting times.

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CHAPTER XVI

THE CONTROL OF THE BLOOD CHEMISTRY IN RENAL AND GASTRO-INTESTINAL DISEASES

CERTAIN disturbances in the alimentary canal such as ulceration and new growth, with or without vomiting or obstruction, and of the urinary tract such as chronic nephritis, blockage of renal tubules and other obstruction to urinary outflow, produce changes in the salt and bicarbonate content of the blood and in distribution and content of body water, which tend to dehydration and to failure in excretion of waste nitrogenous substances. In all of them, there may develop a toxic state closely akin to the true uraemia that occurs in the late stages of progressive chronic nephritis. With appropriate treatment, this toxic state is a crisis from which the body may recover; without such treatment, a fatal termination is to be expected. In the true uraemia of advanced chronic nephritis death can only be postponed.

The purpose of this chapter is to give an account of the principles and control of this appropriate treatment.

Factors involved in the Development of the Toxic State: The toxic state is essentially one of failure of renal function brought about by a disturbance of the availability and distribution of water, bases, chloride and bicarbonate needed by the kidneys for proper functioning. In dehydration (inadequacy of body water), in alkalosis (high plasma bicarbonate content) and acidosis (low plasma bicarbonate content), and in chloride depletion (low plasma chloride content), renal elimination of toxic waste products becomes increasingly difficult. In any given case, one, two or three of these factors may be operating at any time to produce renal failure.

Dehydration and base-chloride depletion may be the result of loss of water, bases and chloride by polyuria, vomiting, or excessive sweating, with or without decreased intake and absorption. Alkalosis is most commonly caused by loss of acid through excessive vomiting, or by excessive intake of sodium bicarbonate or other alkali, whilst acidosis is usually the result of the retention of acid metabolites, diarrhoea, or medication with acid-forming salts as in mandelic acid therapy. Owing to the body's natural tendency to conserve the blood bases, there is usually a reciprocal relationship between the plasma chlorides and the plasma bicarbonate: a high plasma bicarbonate content is usually accompanied by a low plasma

chloride content, and vice versa. When the plasma chloride level is changed by treatment or naturally, the plasma bicarbonate level moves in an opposite direction. This reciprocity is modified in established renal failure when blood bases are lost and both plasma chlorides and bicarbonate are likely to be low.

Principles of Treatment of the Toxic State: Treatment is directed to restoring the body's water content and blood chemistry to normal ranges. In dehydration, water is given as physiological saline or Ringer's solution, or some dilution of these with glucose added. In base and chloride depletion, which is commonly associated with a high plasma bicarbonate level, base and chloride are supplied as physiological saline, Ringer's solution or double strength saline. Frequently, normal saline or Ringer's solution raises the plasma chloride level only slowly or merely prevents further chloride depletion, recourse to double strength saline is then necessary. In acidosis (or bicarbonate depletion), sodium bicarbonate may be given, preferably with sodium chloride to compensate for the fall in the plasma chloride level that occurs as the plasma bicarbonate level rises. In alkalosis (plasma bicarbonate in excess), it is usually sufficient to treat the concomitant dehydration and base and chloride depletion, when the plasma bicarbonate level will fall as the plasma chloride level rises.

Quantitative Considerations: An adult in normal water balance needs about three litres of water a day; and more in the presence of dehydration. The whole of this, in the form of appropriate salt solutions may be given intravenously by slow drip if oral administration is contra-indicated. A "balance" record of fluid intake and output in all forms should be carefully kept, due allowance being made for the water lost through the lungs and skin and in the faeces. In the early stages of treating dehydration there will be marked water retention.

The amounts of sodium chloride and sodium bicarbonate required depend on the concentrations of these substances in the plasma as determined by laboratory tests. A rough calculation of the total sodium chloride requirement is made and the amount is given in the manner best suited to the case.

When the true plasma chloride content is expressed in terms of milli-normal or milli-equivalents per litre, say $[Cl]_p$, then the sodium chloride requirement of an adult expressed as litres of normal physiological saline or normal Ringer's solution is

$$\frac{100 - [Cl]_p}{4}$$

(The mean normal plasma chloride content is 100 milli-equivalents per litre.) Assuming a normal body water content of 40 litres, then sodium chloride deficiency

$$= (100 - [\text{Cl}]_r) 40 \text{ milli-equivalents}$$

$$= (100 - [\text{Cl}]_r) \times \frac{40 \times 58.5}{9000} \text{ litres of physiological saline}$$

$$= (\text{in round figures}) \frac{100 - [\text{Cl}]_r}{4} \text{ litres of physiological saline.}$$

58.5 is the milli-equivalent weight of NaCl and physiological saline contains 9000 mg NaCl per litre.)

If the plasma chloride content is expressed as mg. NaCl per 100 ml., say $[\text{Cl}]_p$, then the sodium chloride deficiency for practical purposes, can be expressed as $\frac{4(590 - [\text{Cl}]_p)}{100}$ litres of physiological saline.

The results of the above calculations should be adjusted for body weights differing markedly from 70 Kg.

Gross base-chloride deficiency can be quickly corrected by the intravenous infusion of twice-normal saline, but if there is also gross dehydration—and there usually is—it is better to start with normal saline or Ringer's solution, and then to give twice-normal saline when the dehydration is reduced. When the adult body is in chloride balance, the kidneys deal with 13-15 g. of sodium chloride per day. Due regard must be paid to this figure when a patient in chloride balance has to continue with intravenous salines. When the patient is a child, it must be remembered that children, and premature infants especially, have poor powers of chloride excretion.

The adjustment of the dosage of sodium bicarbonate depends on repeated determinations of the plasma alkali reserve. In acidosis, the common initial dose is 16 g. a day in 4 doses at four-hourly intervals, given orally, this being adjusted later as found necessary. In cases with permanent renal damage, a maintenance dose will have to be found by the same process. In some cases, especially of renal damage from haemorrhage, incompatible blood transfusion and "crush" injuries, when it is desired to raise the alkali reserve quickly and give water as well, recourse is had to the intravenous infusion of 2 per cent. sodium bicarbonate solution.

Many cases show a deficiency of serum calcium, which may be aggravated if intravenous treatment has to be prolonged. Ringer's solution is therefore to be preferred to plain saline. Glucose, too, is necessary when oral intake is restricted. This can be conveniently

given intravenously as a 5 per cent. solution in fifth-normal or third-normal Ringer's solution. A patient in water and chloride balance can be kept going for several days on a daily input of two litres of 5 per cent. glucose in fifth-normal or third-normal Ringer's solution and one litre of normal Ringer's solution. This gives 12.6 or 15 g. of sodium chloride respectively, 100 g. of glucose and 3 litres of water a day. An example of a case of alkalosis treated by this method is shown in Fig. 6.

Technique of Administration: The administration of saline solutions demands use of the intravenous route since the large volumes of solutions needed render the oral route impracticable. For clinical reasons oral administration is usually undesirable, if not impossible; a few patients can tolerate more than 10 g. of sodium chloride a day when taken by mouth, and this amount is far short of that often needed, particularly at the start of treatment. On the other hand, sodium bicarbonate is best given by mouth because when given in solution intravenously it tends to cause oedema, especially of the lungs. Most patients easily tolerate 16 g. a day in 4 g. doses. Should it cause vomiting, the acid lost in the vomit leaves an equivalent amount of bicarbonate in the blood. Continued vomiting may, however, also entail a loss of blood bases.

Except for the administration of small amounts of water, the rectal route is useless. It is especially ineffective for raising the plasma chloride and bicarbonate levels. Moreover, even physiological saline is apt to cause a good deal of local irritation when given rectally; fifth-normal is the strongest salt solution well tolerated by the colon.

Prolonged intravenous infusions are best given through a cannula tied in a vein of the lower limb. For short periods, a needle inserted into a vein and fixed to the skin by adhesive tape suffices. Double strength saline is best given by a needle into flowing blood to avoid irritation and possible thrombosis of the vein. Light splinting of the limb used is helpful.

It is imperative to leave a good vein intact for withdrawal of blood samples. If this be overlooked treatment may break down because the necessary laboratory tests cannot be made. Only by adequate laboratory control can precision in treatment be attained.

Laboratory Tests Necessary. At the outset of treatment, the blood urea, the true plasma or serum chloride and bicarbonate contents should be determined and repeated daily until treatment

is becoming stabilized; thereafter the progress of the patient and experience will indicate when tests are needed. Treatment should

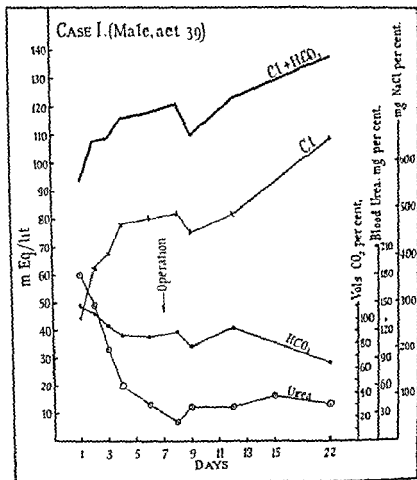


FIG 6—(Case I) *Pyloric obstruction with severe vomiting.* Intravenous therapy extended over 11 days, during which nothing was given by mouth. Treatment was by varying proportions of normal saline and 5 per cent glucose in fifth-normal saline, giving a total of about 2 litres of fluid daily. Twice-normal saline would have given a quicker base and chloride response. Patient made a good recovery. Transfusion with citrated blood after operation induced an attack of tetany. The serum calcium was then 7.1 mg per 100 ml. It fell to 6.0 on day 13, without tetany, recovering to 8.5 on day 22.

not be undertaken or changed without up-to-the-moment knowledge of the blood chemistry. Initial and recurrent erythrocyte counts, and haemoglobin and haematocrit determinations are useful

to keep track of blood volume changes and to determine the presence or absence of anaemia before operation is undertaken.

To avoid the changes which occur through loss of CO_2 when blood is exposed to air, care should be taken wherever possible to collect blood out of contact with air and with minimum stasis: details for doing this are given on p. 212.

For the chemical estimations, any reliable methods may be used. For the bicarbonate estimation the titrimetric method of van Slyke (see "Practical Physiological Chemistry," Hawk and Bergeim, 11th edition, p. 507), modified by using conical flasks and placing the acidified plasma in a 37° incubator for 20–30 minutes to ensure that all the CO_2 has been driven off, has been found most useful, especially when a number of estimations have to be done together. This method has the added advantage of giving an indication of the pH of the plasma. Jaundice and haemolysis make the method less easy, but unless gross do not interfere.

Results are preferably expressed, not in mg. per 100 ml. and vols. CO_2 per cent., but as milli-equivalents per litre, *i.e.* in terms of milli-normality. In the types of cases under consideration, the most important factor is the level of the total plasma base. This is largely accounted for by the bases combined as chloride and bicarbonate, and the sum of these can only be obtained if chloride and bicarbonate levels are expressed as milli-equivalents. The normal ranges are as follows:

plasma chloride	. . .	95 to 105 m.Eq./lit.
plasma bicarbonate	. . .	25 to 35 m.Eq./lit.
chloride plus bicarbonate	. . .	120 to 140 m.Eq./lit.

Tests on the urine, for reaction and chloride content, are not to be relied on as guides to treatment. In the types of case under discussion, the degree of renal failure may be such that the kidney has ceased to respond to changes in plasma base level and reaction. It is the composition of the blood, not of the urine, that determines the well-being or otherwise of the body. It is a general experience to meet patients whose urinary pH does not alter in response to acidifying or alkalizing salts; some patients develop severe acidosis when given ammonium chloride and others alkalosis when treated with alkalis, the probable cause in both types of case being selective renal incapacity. Since the total base in the plasma is a determining factor in renal function, along with the distribution of base as between chloride and bicarbonate, it is essential to determine both bicarbonate and chloride in the plasma.

General Considerations

During intravenous infusion, careful watch must be kept for failure of the infusion, for leaks into the tissues, inflammation and thrombosis of veins, for oedema, and on the lungs and cardiovascular system. In particular, pulmonary oedema must be most carefully watched for, and infusion stopped at once on its appearance. With care in technique, even a failing heart need not deter intravenous therapy, but the addition of fluid-loading of the lungs must do so.

Saline solutions weaker than 100 m.Eq./lit. (0.585 g. NaCl per 100 ml.), the normal chloride strength of the plasma, should be used with reluctance and preferably only when the plasma base and chloride are near, at or above normal levels. Fifth-normal physiological saline, containing approximately 31 m.Eq./lit., used as a vehicle for glucose, tends to abate the restoration of plasma base and chloride. (See case II, days 0 and 1.) The base and chloride levels in the plasma in case I would have been better restored had a solution of salt stronger than fifth-normal been used to administer glucose.

The rectal route is useless for introducing anything other than water in relatively small amounts into the body. The fall in plasma chloride in case II, day 3, and in plasma bicarbonate in case III, day 6, followed attempts to administer salt and bicarbonate solutions respectively per rectum.

In alkalosis, which is usually accompanied by chloride loss and base depletion, adequate administration of base and chloride, as sodium chloride solution, is usually sufficient to restore the normal relations of base-chloride-bicarbonate in the plasma. When renal excretion is restored, alkaline urine is excreted and this helps to remove the excess of alkali in the blood. Administration of acidifying salts by mouth is unnecessary and undesirable. The normal production of acid metabolites in the body helps naturally to correct alkalosis, especially if neutral base is supplied. On the other hand, because of the natural production of acid metabolites, acidosis usually needs alkali to correct it.

When alkali is needed, sodium bicarbonate, the main physiological alkali of the blood, is usually easily given by mouth and best controlled when so given. The response to oral administration is so quick and effective (see case III, days 8-10) that intravenous administration is only needed in special cases (e.g. case II, days 0-1, 5). The intravenous use of alkalizing salts such as sodium

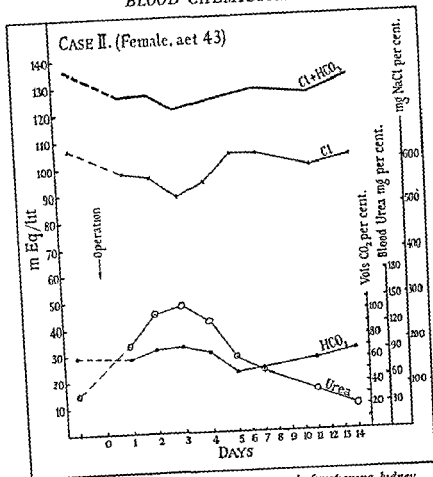


FIG 7—(Case II) Nephrolithotomy on sole functioning kidney.

SUMMARY OF TREATMENT

SUMMARY OF TREATMENT										
Substance	Route	Days								
		0	1	2	3	4	5	6-12	13	14
NaCl (g)	intraven	11.6	10.3	—	18	27	19.4	—	—	—
" "	oral	—	—	—	—	—	—	3.8	3.8	1.5
" "	rectal	—	—	15.3	7.5	—	—	—	—	—
NaHCO ₃ (g)	intraven	10	14	—	—	—	10	—	—	—
" "	oral	—	—	12	12	8	12	16	—	—
Water (ml)	intraven	4,180	2,450	—	2,000	3,000	1,700	—	—	—

Intravenous sodium bicarbonate was used to make the urine alkaline and ensure clearance of intra-renal bleeding. There was a dangerous rise of blood urea in day 3 associated with the fall in the plasma chloride following rectal administration of saline. Intravenous saline reversed this state. Urine volume, 170 ml. on the day of operation, rose to 1830 ml. on the second day after operation. The urea clearance values were 28 per cent of normal before and 34 per cent of normal 14 days after operation.

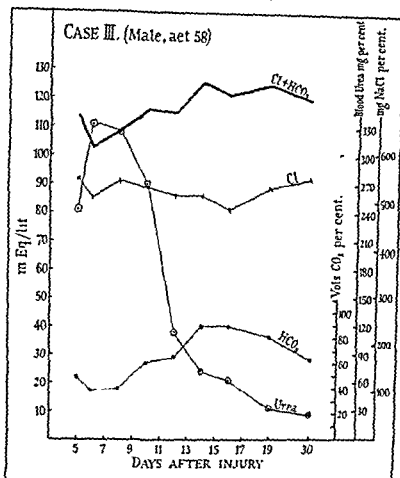


FIG. 8 — (Case III) Traumatic ("crush injury") renal failure.

SUMMARY OF TREATMENT

Substance	Route	Days					
		5	6	7	8	9	10-14
NaCl (g)	intravenous	—	9	1.8	—	—	—
NaHCO ₃ (g)	oral	—	—	—	16	20	12
" "	rectal	20	—	—	—	—	—
Water (ml)	intravenous	—	1,000	1,000	—	—	—

Bicarbonate by rectum did not prevent the fall in the plasma bicarbonate. The serious degree of renal failure was associated with low values of plasma base, chloride and bicarbonate. Urine volume fell to 200 ml. on day 4, but rose to 2,230 ml. on day 6. Urine volume fell again on day 6. checked the rise in blood urea on day 6 till the plasma bicarbonate rose.

Intravenous therapy had to be discontinued because severe pulmonary oedema developed on day 7. Urea clearance values rose from 9 per cent. of normal on day 9 to 57 per cent. on day 16. Patient recovered and withstood well amputation of his injured lower limb.

lactate or gluconate is similarly unnecessary. The oral dose of sodium bicarbonate may be given as a draught containing about one quarter as much sodium chloride as bicarbonate.

The method of treatment outlined holds out hopes of tiding patients over crises that would otherwise prove fatal. But, in addition, it is a valuable adjunct in abdominal surgery, both in preparing a patient for operation and in post-operative treatment. The routine determination of plasma chloride and bicarbonate levels and their adjustment if necessary before operations on the alimentary tract in the abdomen and their follow-up after operation, with treatment as indicated, are invaluable. The frequent loss of plasma base and chloride after operation on the gut may not be rectified without assistance; prompt detection of this failure is best attained by routine post-operative determinations of plasma chloride and bicarbonate so that treatment can be started early. This also applies to operations on the urinary tract.

Summary

Dehydration is treated by intravenous infusion of normal saline or Ringer's solution; chloride depletion by normal saline, Ringer's solution or double strength saline, also given intravenously; plasma bicarbonate depletion by administration of sodium bicarbonate given orally for choice, but in selected urgent cases, *e.g.* in renal tubular damage or blockage, intravenously; alkalosis by intravenous infusion of saline solutions depending on the co-existence of dehydration base and chloride depletion. The oral route is used when results can be obtained by it. In all cases treatment is controlled and guided by laboratory determinations of the blood urea, plasma chlorides and bicarbonate.

W. W. KAY.

CHAPTER XVII

EXCRETION TESTS IN ADDISON'S DISEASE

IN this article, Addison's Disease (adrenal cortical insufficiency) will be dealt with only so far as the diagnosis can be established or excluded with the aid of laboratory methods. A test is required to help the clinician either to confirm or to rule out this diagnosis; an incorrect diagnosis of Addison's disease is frequently made in cases of fatigue states, with low blood pressure, which are labelled as subclinical adrenal deficiencies unnecessarily treated with potent adrenal cortical extracts.

Robinson, Power and Kepler Water Excretion Test

Robinson, Power and Kepler (1941) from the Mayo Clinic, have worked out two procedures which can easily be performed in hospitals during 24 hours and in laboratories where common routine biochemistry is done. These tests give a striking aid in diagnosis. There are two types of patients whose further investigation may be required: (1) patients showing varying degrees of symptoms of Addison's disease: weakness, pigmentation, hypotension, loss of weight, anorexia, gastrointestinal disturbances and (2) patients chronically tired, with low blood pressure, in whom it is necessary to show that the adrenal cortices are functionally normal. There is usually no metabolic or anatomical cause for the symptoms which must be regarded as "functional."

The physiological basis of this test, which is a simplification of the more elaborate Cutler, Power and Wilder test (see p. 172) is as follows. Following rapid intake of a considerable quantity of water, patients with Addison's disease usually do not show normal diuresis. *Eventually they excrete the excess of water, but the time required is prolonged. Stated differently, in most cases of Addison's disease, the kidneys continue to excrete fairly concentrated urine after water has been ingested.* (See Procedure I.)

Patients suffering from Addison's disease tend to excrete excessive amounts of NaCl but to retain urica. This observation is made use of in Procedure II.

Instructions to the Ward: No special salt restriction is necessary before the test, but if the patient is being treated with desoxycorticosterone acetate, or potent adrenal cortical extracts, these

must be discontinued for at least two days before the test. Beware of Addison's crisis.

Day before the Test Patient eats three ordinary meals without extra salt. He may drink water as desired. Nothing is to be eaten or drunk after 6 P.M. At 10.30 P.M. the bladder is emptied and the urine discarded.

Test : Collect all urine voided from 10.30 P.M. until and including 7.30 A.M. Label and mark "night urine." Measure the volume and save it for chemical analysis (Procedure II), if that should be necessary later. Breakfast is omitted. At 8.15 A.M. draw blood from the patient (fasting) for plasma urea and chloride estimations. The patient is asked to void urine again at 8.30 A.M. and immediately thereafter he is given 20 ml. water per Kg. body weight (9 ml. per pound). He is asked to drink this within the next 45 minutes. At 9.30, 10.30, 11.30 A.M. and 12.30 P.M. he is requested to empty his bladder, each specimen is kept in a separate container and labelled. The volume of the largest of these four specimens should be measured. The patient should be kept at rest in bed except when up to void urine.

Interpretation. Procedure I. "The Water Test." (Based on volume of urine.) (1) If the volume of any single hourly specimen voided during the morning is greater than the volume of the "night urine" the result indicates the absence of Addison's Disease. Robinson, Power and Kepler (1941) did not encounter any exception to this rule.

(2) If the volume of the largest hourly specimen voided during the morning is less than the volume of the "night urine," the response to the test is positive, that is, Addison's disease may or may not be present. To establish the diagnosis Procedure II should be carried out.

Procedure II. (Based on chemistry of blood and urine.) Analyze the plasma and the "night urine" for their contents of urea and chloride. From these determinations the value "A" is obtained from the following equation :

$$"A" = \frac{\text{Urea in urine}}{\text{Urea in plasma}} \times \frac{\text{Chloride in plasma}}{\text{Chloride in urine}} \times \frac{\text{Vol. of "day urine"}}{\text{Vol. of "night urine"}}$$

The term "day urine" refers to the *largest* of the hourly specimens voided during the morning, "night urine" to the entire amount from 10.30 P.M. to 7.30 A.M. Express all values in mg. per cent.

If the value of "A" is greater than 30, the patient probably is not suffering from Addison's disease.

If the value is less than 25, the patient, provided that nephritis is excluded, probably has Addison's disease.

If the result is doubtful, the test devised by Cutler, Power and Wilder (1938) and Willson *et al.* (1942), may follow immediately. None of the patient's time is wasted since the day of the "water test" constitutes the first day of the provocative test. Robinson, Power and Kepler encountered in their series of cases (38 of Addison's disease, 50 miscellaneous) only two instances in which it was necessary to resort to the Cutler, Power and Wilder test. They encountered low ratios in cases of nephritis, diabetes insipidus and in a case of dehydration and fever.

Cutler, Power and Wilder Test

The Cutler, Power and Wilder test is based on the concentration of the urinary chloride after the intake of Na, K and water has been controlled for a period of three days. It must be carried out in hospital and frequently makes the patient with Addison's disease so ill, that it must be terminated before completion. On the day preceding the first day of the standardized examination the patients are served a general diet, but are given no extra sodium chloride. (Discontinue extra sodium chloride even earlier.)

Test : For three days a standard diet is given containing 0.95 g. of Cl-ion, 0.59 g. of Na and 4.1 g. of K daily.

Owing to variation in the foods available in different countries, the detailed diet of Cutler *et al.* is not reproduced here. Essentially the diet should contain moderate amounts, say 110 g. daily of salt free bread, 30 g. salt-free butter, cream, milk 200 g. only, in coffee, and fairly liberal amounts of potatoes, other vegetables and fruits. All cooking to be carried out without salt. Free fluid intake is encouraged on the first day, on the afternoon of which extra potassium (as potassium citrate) is given in a dosage of 33 mg. K per Kg. bodyweight and the same dose of potassium citrate is repeated in the morning.

On the third day 20 ml. of fluid per Kg. are given before 11.00 A.M. The procedure is finished at noon on the third day and the urine of the last 4 hours of the 52-hour period is chemically examined. In some cases it is useful to extend the deprivation of salt in order to obtain additional evidence regarding the degree of adrenal function.

Interpretation : Usually chloride estimations will give the necessary information, but if the chloride result is doubtful, sodium estimations may be of further assistance.

The chloride range found for normals was 17-141 mg./100 ml. with a mean of 55 mg. and in patients with Addison's disease 229-356 mg./100 ml. with a mean of 293; subsequent workers have not always found such a clear-cut distinction; Paschkis and Price (1944) studied 50 patients, 27 of whom suffered from asthenia, and found a chloride level of more than 125 mg./100 ml. in 13 of them, and more than 225 mg. in 4, none of whom had Addison's disease. They stress the considerable variations which may be found when the test is repeated on the same patient. For practical purposes, a level less than 125 mg./100 ml. may be regarded as excluding Addison's disease, and one of over 225 mg./100 ml. as strongly supporting the diagnosis. If an intermediate value is found, a continuation of the period of salt restriction with additional potassium up to 9 g. per day sometimes gives conclusive results, but is dangerous on account of the risk of producing a crisis from which the patient may not recover. There seems less overlap when sodium values are estimated (Willson *et al.*, 1942; Dryerre, 1939). The normal range is from 6-85 mg./100 ml. with a mean of 22.4, the values for Addisonian patients being 165-282 mg./100 ml. with a mean of 207.

Under the conditions named the concentration of Cl in the urine of the morning of the third day of the period of the deprivation of salt was diagnostically more significant than the volume of urine. A patient with Addison's disease will usually excrete a relatively small volume of urine, but with it as much as, or more Na and Cl than would be excreted under similar conditions by the subject with normal adrenal function (Willson *et al.*, 1942). The concentration of K in the urine, the total excretion of Na, Cl, or K and the values or changes in values of the Na, Cl, or K of the blood plasma are of less consistent value. The serum Na, Cl, K of patients with Addison's disease on normal diet are sometimes within normal limits except during periods of crisis or in terminal stages. The test also provides more dependable information than any of the symptoms of adrenal insufficiency encountered during a three-day period of restriction of salt. Dryerre (1939) and also Willson *et al.* (1942) showed that patients with Addison's disease, who are subjected to salt restriction but receive desoxycorticosterone acetate respond more like normal subjects to the Cutler, Power, and Wilder test.

Dangers of Test: It is emphasized that the Cutler, Power and Wilder provocative test should only be used if the clinician is prepared to recognize and treat acute adrenal insufficiency if it

should occur. At the close of every examination of subjects suspected of Addison's disease, there should be given intravenously: 1 litre of a solution containing 50 g. dextrose, 10 g. NaCl, 5 g. Na-citrate plus 20 ml. of cortical extract. This fluid should also be ready in cases of emergency, which may occur even though there is less risk than in the formerly used salt restriction tests over a 6-day period (Harrop *et al.*, 1933 ; Wilder *et al.*, 1937).

As an example of patients investigated with the Robinson, Power and Kepler test a case of Addison's disease without pigmentation may be cited. The diagnosis made on grounds of positive tests was confirmed at necropsy. This female patient had chronic pulmonary tuberculosis but the destruction of the cortices of both suprarenals was due to secondaries from a carcinoma of the breast also metastasized into the lungs, brain and lymph glands (radical mastectomy 12 years previously).

Her symptoms were weakness, fainting attacks and low blood-pressure. The "water-test" gave for the night urine a volume of 805 ml., the largest hourly morning specimen had a volume of 39 ml. and the value of "A" was 4.5. (Plasma urea 31 mg./100 ml., plasma chloride 560 mg./100 ml. "Night urine" urea 760 mg./100 ml., chloride 146 mg./100 ml.)

J. H. WAELSCH.

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CHAPTER XVIII

BIOCHEMICAL AIDS IN THE DIAGNOSIS OF NUTRITIONAL DEFICIENCIES

IN the diagnosis of nutritional deficiency in a patient three procedures are essential: a clinical examination, a dietary history and a medical history. None of these by itself is sufficient, and none can be omitted. But even this triad is usually inadequate and must be supplemented by biochemical aids. No one should diagnose deficiency of vitamin A on a history of night-blindness, or on a history of a diet apparently low in vitamin A and its precursors; and there are no specific clinical signs of this or almost any other deficiency except in the late stages of disease. Therefore, even for the diagnosis of advanced nutritional deficiency, biochemical aids should be sought. These biochemical tools have three uses: as an aid to clinical diagnosis, in nutritional surveys for public health purposes, and in research; they are needed by the clinical pathologist for the first purpose.

An important function of the clinical pathologist is to reveal nutritional deficiencies unsuspected by the clinician. Such deficiencies are seldom single even though the signs may be characteristic of one of the classical diseases: pellagrins, treated with niacin, frequently develop the signs of deficiency of riboflavin; the lure of a classical case of scurvy may obscure a simultaneous deficiency of vitamin A. And now, when specific therapy with synthetic vitamins is easy, the importance of obtaining information about concomitant but less dramatic deficiencies has become greater. Further, deficiency may arise from unsuspected causes: from destruction of nutrients in or poor absorption from the gut, from inadequate bacterial synthesis in the gut, from faulty metabolism such as failure of phosphorylation of a vitamin to give a coenzyme, from increased excretion, or from increased requirement.

Because nutritional deficiency is usually multiple, it is an advantage to be able to make with ease and rapidity various estimations with one sample of blood or one urinary tolerance test. A sample of blood can conveniently be used in the following way: 20 ml., drawn without stasis, are added to a graduated tube containing 24 mg. of ammonium oxalate and 16 mg. of potassium oxalate; for the estimation of pyruvate about 0.2 ml. is added direct from the syringe to a tared tube containing 2 ml. of ice-cold 10 per

cent. trichloroacetic acid, and the tube is reweighed; 1 ml. of the oxalated blood is used for the determination of haemoglobin, for the haematocrit, and, if these are required, for the erythrocyte and leucocyte counts and the sedimentation rate; 1 ml. is used for ascorbic acid and 0.6 ml. for riboflavin (if desired). The remaining 17 ml. are centrifuged in a constricted tube (Butler and Cushman, 1940). The plasma is used for the determination of phosphatase (2 ml.), prothrombase clotting time (0.5 ml.), vitamin A and carotene (2 ml.), and protein by the densitometric and micro-Kjeldahl or biuret methods (0.7 ml.). The leucocyte-platelet layer is weighed (usually 80-120 mg.), laked with water, and used for the determinations of any of the following: ascorbic acid (20 mg.), thiamine (50 mg.), riboflavin (25 mg.), and niacin (25 mg.). The erythrocytes can also be used, if desired, for determinations of niacin and of ascorbic acid.

Even though no water-soluble vitamins are determined in plasma by this method, it is desirable to draw the sample of blood in the fasting state before breakfast, and this is essential if a test-dose is to be given for a tolerance test. In this case, the patient should empty his bladder soon after waking; urine should be collected over the next 1-2 hours during which time the blood sample is also taken; the test-dose containing ascorbic acid, thiamine, niacinamide and riboflavin is then drunk, the patient has breakfast, and all urine passed during the next four and subsequent three hours is collected.

In the case of most of the determinations insufficient critical work has as yet been done to define their limitations and interpret their results. In all cases, however, the interpretation of the biochemical tests must be made in relation to the other evidence, for they are but part of the diagnosis.

In the account that follows, details of methods that are being used by the Oxford Nutrition Survey are set out, without any claim to originality, since many of the clinical pathologists in Europe for whom this book is intended will not have easy access to the recent literature. A discussion of this literature and of the merits or demerits of methods is intentionally excluded, and limitations of space prevent an adequate discussion of the interpretation of results. The author hopes that those who have suggestions or experience of these or other methods will correspond with him. Certain estimations, such as of phosphatase and prothrombase clotting-time, are discussed elsewhere in this book.

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Ltd., Grade A); one lixiviating jar to form a water-jacket for the cylinder; six 100 ml. volumetric flasks; one 300 ml. separating funnel with the end tapered and bent to form a U; Pasteur pipettes, rubber tube and mouthpiece; filter-papers; copper wire (gauge 18, about 50 cm. long); clock or watch reading in minutes; hydrometer (0.99 to 1.06), fine sand.

Reagents: 350 ml. of paraffin ($D_{20}^{20} = 0.79$); 200 ml. of bromobenzene ($D_{20}^{20} = 1.49$); 500 g. of paraffin wax. Technical grades of purity are sufficient.

Standard Sodium Chloride Solutions: Pure sodium chloride is dried at dull red heat in a vitreosil crucible for 2 hours and allowed to cool in a desiccator, copper sulphate standards may alternatively be used. The following six solutions are separately prepared, the salt being weighed to 0.1 mg. and made up in an accurate volumetric flask immersed in a water bath at 20° C.

NaCl (g / 100 ml)	D_{20}^{20}	NaCl (g / 100 ml)	D_{20}^{20}
2.5	1.01757	4.0	1.02789
3.0	1.02102	4.5	1.03130
3.5	1.02447	5.0	1.03471

These solutions will keep at 0° C. in rubber-stoppered bottles for several months, small quantities being removed to smaller bottles for routine use and checked occasionally against the stock solutions.

Method: The measuring cylinder is fixed inside the jar with paraffin wax at its base and is surrounded with water containing a little CuSO_4 to prevent the growth of algae; the water is covered with paraffin wax. A heavy solution, $D_{20}^{20} = 1.050$, is prepared by mixing 158 ml. of paraffin and 92 ml. of bromobenzene; after its density has been checked with the hydrometer, the solution is poured into the cylinder. A light solution, $D_{20}^{20} = 1.000$, is prepared by mixing 175 ml. of paraffin and 75 ml. of bromobenzene; after its density (which, as in the case of the heavy solution, should be within ± 0.002 of the given value) has been checked, the solution is carefully introduced from the separating funnel into the cylinder to form a layer above the heavy solution. After the cylinder has stood for two or three hours for temperature equilibration, the two layers are carefully mixed using a wire stirrer which should fit the cylinder fairly closely to eliminate lateral movements. Mixing is begun at the line of juncture of the two solutions by raising and lowering the wire slowly with an excursion of about "50 ml." on the cylinder scale, the amplitude being increased by steps of "50 ml" above and below until the full depth of the solution is covered by each stroke; forty strokes at each amplitude usually complete the

mixing which is indicated by a distance of about "50 ml." between any two adjacent drops of the standard solutions added to the cylinder.

The gradient is calibrated by introducing into it a drop of each of the standard solutions by means of a capillary pipette, the drop being nearly blown out of the pipette just below the surface of the gradient and the pipette then withdrawn. The positions after 2 minutes in the gradient are plotted against the densities of the solutions, and a calibration curve is obtained; this is nearly linear.

A drop of plasma or serum is added in the same way, and after up to 100 drops have been introduced a further set of standard drops is added; it is desirable, though not essential, to do each estimation in duplicate. The solution is then cleaned by sprinkling a little sand. The gradient may be used repeatedly for several months, but should be tightly stoppered when not in use to prevent evaporation of bromobenzene. Turbidity may be removed by sprinkling with CaCl_2 ground in a few ml. of the upper mixture.

Calculation: The relation between density of plasma or serum and protein content is given by the following equation (Phillips, Van Slyke *et al.*, 1945):

$$\text{Protein in g. per 100 ml.} = 360(\text{density} - 1.0070).$$

If plasma is used, care must be taken to avoid haemolysis; heparin or Heller and Paul's oxalate mixture should be used (3 parts ammonium to 2 parts potassium oxalate, 0.20 g. per 100 ml. of blood), and with the latter 0.0010 must be subtracted from the density to allow for the density increment due to the oxalate.

HAEMOGLOBIN

The method of Linderström-Lang may be used as described above for measuring blood density with the following modifications. The two solutions consist of: light, $D_{20}^{20} = 1.025$, 33 per cent. bromobenzene, 67 per cent. paraffin; heavy, $D_{20}^{20} = 1.100$, 44 per cent. bromobenzene, 56 per cent. paraffin. A hydrometer reading to 1.13 is needed. The sodium chloride standards are:

NaCl (g./100 ml.)	D_{20}^{20}	NaCl(g./100ml.)	D_{20}^{20}
5	1.03471	9	1.06142
6	1.04146	10	1.06798
7	1.04816	11	1.07449
8	1.05481		

The blood must be very carefully mixed immediately before the drop is introduced into the gradient. The density of plasma must also be measured, and the equations derived by Phillips, Van Slyke *et al.*, for their copper sulphate method are used for obtaining haemoglobin concentration and haematocrit from whole blood and plasma density.

VITAMIN A AND CAROTENE

Vitamin A may be estimated in serum spectroscopically, but the commoner method uses the Carr-Price reaction in which a colour (maximum at 620 m μ for vitamin A and 590 m μ for carotene) is developed by reaction with antimony trichloride and chloroform (Dann and Evelyn, 1938; Clausen and McCoord, 1938; Kimble, 1939; May *et al.*, 1940; Yudkin, 1941; Hsu, 1942). When vitamin A is taken by mouth its esters are hydrolysed and absorption in the form of the alcohol starts very soon; the concentration in plasma reaches a peak after about four hours and returns to normal slowly; absorption is greatly assisted by the presence of bile salts. The normal amount in adults is about 70–150 I.U. per 100 ml. of serum, and when the value falls below 30 poor dark-adaptation tends to develop; in children, however, lower values are normally found. In estimations made by the Oxford Nutrition Survey during the past three years upon 2,195 subjects, excluding children, from various normal population groups in England, the mean value for vitamin A has been 86 I.U. per 100 ml. of serum, the S.D. being 31 and the range < 10 to 245. Carotenoids in serum are more variable and only about half those estimated by the Carr-Price reaction are provitamins A; the corresponding figures obtained by the Oxford Nutrition Survey on 2,291 subjects are: mean, 94 μ g. per 100 ml. of serum, S.D., 42; range, < 6 to 312. If vitamin A and its precursors are rigidly excluded from the diet for months there still remain about 20 μ g. of carotenoids (expressed as β -carotene) per 100 ml. of serum, mostly consisting of xanthophyll.

In conditions in which fat is poorly used by the body, such as coeliac disease or cystic disease of the pancreas, vitamin A is poorly absorbed; this may be tested by giving a test-dose of 10,000 I.U. per Kg. of body-weight, and estimating vitamin A in serum after 4 and 7 hours. Loss also occurs in the faeces if liquid paraffin is taken in large doses. Low blood values may be produced by factors other than diminished ingestion or poor absorption of the vitamin and its precursors: by poor conversion of carotene to

vitamin A in the liver, as apparently occurs in diabetes mellitus ; by poor storage of the vitamin in the liver, as is found in cirrhosis ; or by increased destruction or renal excretion, as in fever or chronic nephritis.

The level of vitamin A in the blood of an individual is relatively constant despite wide variation in intake, provided a few days are allowed to discount the effects of a temporary surfeit. When healthy volunteers are placed on diets deficient in vitamin A and carotenoids, the amount of the former falls slowly, and of the latter rapidly. The exact rate at which serum vitamin A falls and dark-adaptation deteriorates has varied in an as yet inexplicable way in different experiments ; significant changes have apparently been obtained at times in a matter of days, and at other times only after many months. The response of serum vitamin A and of poor dark-adaptation due to deficiency, in subjects treated with massive doses of vitamin A, also varies ; sometimes the values return to normal in a matter of hours, and at other times not for weeks. These different responses seem to be due to unknown differences in the diets used ; they complicate the interpretation of results of the estimation of vitamin A. It may reasonably be concluded that a level of 70 I.U. of vitamin A per 100 ml. of serum excludes a diagnosis of deficiency, whereas a value in adults below 30 I.U. strongly suggests deficiency, particularly in the absence of fever.

Principle: Protein is precipitated in plasma or serum by addition of ethyl alcohol, and the vitamin A and carotenoids are extracted with petrol ether. After the carotenoids have been estimated colorimetrically, the petrol ether is evaporated and the blue colour of vitamin A is produced with the residue by adding chloroform and antimony trichloride. The transient blue colour is estimated, this being due to vitamin A and carotenoids ; by subtraction of the latter the amount of vitamin A is obtained.

Apparatus: Photoelectric colorimeter with 440 m μ and 620 m μ filters. Automatic pipette to deliver 2 ml. in about 2 seconds.

Reagents (For 100 estimations): 300 ml. of ethyl alcohol (95 per cent) ; 500 ml. of petrol ether (B.P. 40–60° C) ; 200 ml. of chloroform ; 300 ml. of Carr-Price reagent (14 per cent. w/w of SbCl₃ in CHCl₃) ; 10 ml. of acetic anhydride.

Method: To 2 ml. of plasma or serum are added slowly an equal volume of alcohol with shaking. After addition of 4 ml. of petrol ether the mixture is shaken vigorously for 10 minutes. After allowing to settle, 2 ml. of the petrol ether layer are removed to a colorimeter

tube, 0.25 ml. of petrol ether is added and the yellow colour estimated using a 440 m μ filter. The tube is then placed in a water-bath at 50–60° C., and the contents are evaporated to dryness (this takes about 20 minutes). The residue is dissolved in 0.2 ml. of dry chloroform and 1 drop of acetic anhydride is added. The tube is replaced in the colorimeter, the 620 m μ filter is inserted, and 2 ml. of Carr-Price reagent are added from an automatic pipette. The maximum deflection, which occurs about 6 seconds after adding the reagent, is noted; the colour is more stable if the reading is made about 0° C., and the estimation must be discarded if the tubes are cloudy. Calibration curves are made for carotene and for vitamin A using, if possible, crystalline β -carotene and crystalline vitamin A alcohol, or a concentrate of known potency. If a fish-oil concentrate is used whose vitamin A potency has been determined spectrographically, the conversion factor 1,600 should be used (Morton, 1942; Irwin, 1944). 1 I.U. of vitamin A is defined as the biological activity of 0.6 μ g. of β -carotene.

THIAMINE (VITAMIN B₁)

The amount of thiamine in the blood is very small and not easily measured chemically. Three different methods have mainly been used. The first makes use of the discovery of Schopfer (1937) that a fungus, *Phycomyces blakesleeana*, will grow only in presence of the vitamin and that it is sensitive to a concentration as low as one part in 10⁷. This method has been used for blood (Meiklejohn, 1937; Sinclair, 1938, 1939) and for cerebro-spinal fluid (Sinclair, 1939). Unfortunately the method is tedious and results take nearly two weeks to obtain because of the time needed for the growth of the fungus. The second method makes use of the physiological function of the vitamin, as cocarboxylase, in the degradation of pyruvate by yeast; this method (Goodhart and Sinclair, 1939, 1940; Goodhart, 1940; Westenbrink *et al.*, 1943) needs Warburg manometers and cannot therefore be easily used for routine work. In the third method the vitamin is oxidised to thiochrome and the blue fluorescence of this compound is estimated; unfortunately this method has not yet been made sufficiently sensitive for routine use, although it has been successfully used with a special fluorimeter (Friedemann and Kmiecik, 1943).

Blood contains about 7.5 μ g. of thiamine hydrochloride per 100 ml., of which only 0.5 μ g. is present in plasma. Most of the total—about 7 μ g.—is present as cocarboxylase which only occurs

in the cells of blood and in highest concentration in the leucocytes. Indeed, the estimation of thiamine in these affords a useful method of assessing the nutriture of a person regarding thiamine, provided one of the three methods mentioned above can be used. The leucocyte-platelet layer normally contains not less than 1 $\mu\text{g.}$ of thiamine hydrochloride per g. and is the most satisfactory part of blood to use for estimations for various reasons. First, it contains a relatively high concentration of the vitamin, and therefore other substances, such as protein, interfere less; it is not necessary to precipitate protein for the cocarboxylase test, nor is it necessary to add control flasks to allow for the presence of interfering substances in the *Phycomyces* test. Secondly, the biologically active compound of thiamine, cocarboxylase, is mainly estimated in leucocytes, since they contain negligible quantities of free thiamine. Thirdly, although if subjects are placed upon a deficient diet the amount of thiamine in whole blood falls before clinical evidence of deficiency appears, the amount in the leucocytes probably begins to fall earlier and therefore this estimation is more valuable; only relatively late in deficiency does the amount in whole blood fall markedly. Laboratories that have not special equipment will probably find the estimation of thiamine in leucocytes by the *Phycomyces* method to be the most valuable, even though the results are delayed by eleven or more days.

Principle: Leucocytes are added to a flask containing a synthetic medium and, after sterilization, the flask is inoculated with a suspension of *Phycomyces blakesleeanus*. Control flasks containing known amounts of thiamine are also inoculated. After the fungus has grown for 10 days, it is removed, washed and dried to constant weight, and the growth in the flask containing leucocytes is compared with that in the control flasks.

Reagents: Glucose; Asparagine; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; KH_2PO_4 .

Method: 4 ml. of medium, composed of glucose (0.84 M), asparagine (0.05 M), MgSO_4 (0.003 M) and KH_2PO_4 (0.002 M) at pH 6.7, are added to each of two 50 ml. Erlenmeyer flasks. 20 mg. of the leucocyte-platelet layer of blood are added to one flask and 30 mg. to the second, and water to bring the final volume to 9 ml. The flasks are sterilized by tyndallization and inoculated with 1 ml. of a suspension of the spores of the fungus grown for a fortnight on Sabouraud's medium with 2 per cent. malt extract. A control set of flasks containing known amounts of thiamine (0, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.3, 0.5, 2.5 $\mu\text{g.}$) is set up in duplicate and treated

similarly. The flasks are kept in the dark at about 18° C. for 10 days, and the fungus is then harvested, washed, dried and weighed.

PYRUVATE

The amount of pyruvate in blood is increased in various conditions such as deficiency of thiamine, exercise, anoxaemia, pregnancy toxæmia, or poisoning with arsenic or phosphorus. The estimation by the hydrazine method is simple, the best modification being that described by Friedemann and Haugen (1943). A latent abnormality may be detected by first administering glucose (Bueding *et al.*, 1941).

NIACIN (Nicotinic acid)

Like thiamine, niacin may be estimated in blood chemically, microbiologically or enzymatically. Most workers have used the cyanogen bromide method in which the solution of niacin is warmed with CNBr in the dark and treated with a primary aromatic amine such as *p*-amino-acetophenone, the yellowish-green colour being estimated (Swaminathan, 1938; Bandier and Hald, 1939; Melnick and Field, 1940); the method is not specific since certain other pyridine derivatives give similar colour tests. In the microbiological method the amount of lactic acid produced by *Lactobacillus arabinosus* is measured (Snell and Wright, 1941). Like thiamine, niacin acts in the body as part of coenzymes, and the activity of these may be estimated if suitable apparatus is available (Axelrod and Elvehjem, 1939; Kohn and Bernheim, 1939; Axelrod *et al.*, 1940; Vilter *et al.*, 1940).

The amount of niacin in the blood of normal persons ranges from 0.52–0.83 mg. per 100 ml. (Melnick *et al.*, 1940). About 95 per cent. is in the erythrocytes mainly in the form of coenzymes, 1 per cent. in the leucocytes, and 4 per cent. in plasma in the form of free niacin (or its amide). Oral administration produces a rise within an hour, but the value returns to normal promptly. There seems to be general agreement that the estimation of niacin in whole blood or urine is useless for the diagnosis of deficiency since normal values may be found in untreated pellagrins (Axelrod *et al.*, 1940; Briggs, 1941; Kochhar, 1941; Field *et al.*, 1941). In animals, however, there is a fall in the coenzyme content of tissues such as liver or muscle during deficiency, and it may therefore prove valuable to estimate niacin by the microbiological method in leucocytes; 10 and 15 mg. are reasonable amounts to add to two assay tubes

At present it seems that some evidence of the nutriture regarding niacin is obtained by giving a test dose of niacinamide and estimating fluorimetrically in the urine passed subsequently one of its derivatives, N^1 -methylnicotinamide or " F_2 " (Najjar and Wood, 1940; Huff and Perlzweig, 1943; Coulson *et al.*, 1944; Najjar, 1944). The method is described below but its value is doubtful.

RIBOFLAVIN

Riboflavin can also be estimated chemically, microbiologically or enzymatically. It produces a characteristic fluorescence spectrum with a maximum at 565 m μ at pH 6, and this property has been used for the estimation of the vitamin in blood and urine.

The vitamin forms part of coenzymes and enzymes in the body, and the concentration in blood of one of these—the d -aminoacid-oxidase—has been used for estimating the vitamin (Klein and Kohn, 1940). The best method for blood, however, is based on the essential nature of riboflavin for the growth of *Lactobacillus helveticus*; this method determines free riboflavin and also that combined as nucleotides (Strong *et al.*, 1941). Unfortunately, however, estimations in whole blood seem to be of little value because the amount remains normal until very late in deficiency (Axelrod *et al.*, 1941). But, as with niacin, the amount of riboflavin in tissues falls, and therefore its estimation in leucocytes is more promising. Blood contains about 30 μ g. of riboflavin per 100 ml., and the leucocyte-platelet layer about 400 μ g. per 100 g., values obtained by the Oxford Nutrition Survey upon normal population groups in this country have given, for blood a mean of 27 μ g. per 100 ml. (number 600, S.D., 18, range, 13–85), and for leucocytes a mean of 418 μ g. per 100 g. (number, 283; S.D., 148, range, 150–920). As insufficient work has been done to establish the variation in the concentration of riboflavin in leucocytes in health and disease, the method of estimation is not here described in detail. It is based on the principle (Snell and Strong, 1939) that if *Lactobacillus helveticus* is grown upon a synthetic medium containing small amounts of riboflavin, its growth (as measured by turbidity) or metabolic activity (as measured by titratable acidity) is linearly related to the concentration of riboflavin over a limited range. The amount of acid produced in tubes containing blood or leucocytes is therefore compared with controls containing known amounts of riboflavin. Convenient amounts of the leucocyte-platelet layer are 10 and 15 mg. in the two assay tubes.

More information, however, has been obtained about the excretion of riboflavin in urine, particularly after a test dose, and the procedure for this is described below.

ASCORBIC ACID

Ascorbic acid, in its reduced form, is usually estimated in plasma or urine by titration with 2 : 6-dichlorophenolindophenol, suggested originally by Tillmans (1927). The dye is reduced by other substances that may be present in blood or urine, such as sulphhydryl compounds or thiosulphate. Titration with methylene-blue in the presence of light has also been used (Lund, 1937; Butler *et al.*, 1943). Since ascorbic acid is readily oxidised to dehydroascorbic acid which then mutarotates to 2 : 3-diketogulonic acid, precautions must be taken if the vitamin is to be estimated in its reduced form : the plasma or urine must be fresh or suitably preserved and haemolysis must be avoided. Further, these methods are not suitable for whole blood or for the leucocyte-platelet layer of blood unless special precautions are taken. Such estimations are desirable for nutritional purposes because the amount of ascorbic acid in plasma reflects the immediate past intake, whereas estimations in whole blood, and particularly in the leucocyte-platelet layer of blood, are less affected by the patient's diet in the preceding few days. Roe's hydrazine method estimates the vitamin after oxidation and therefore can be used on samples of blood kept at room temperature for a day or two or on acidified urine kept for longer periods ; it is also more convenient than the other methods (Roe and Kuether, 1943 ; Lloyd *et al.*, 1945).

The amount of ascorbic acid in plasma drawn at least several hours after the last ingestion of the vitamin varies from 0 to about 1.7 mg. per 100 ml., the renal threshold being reached at about the latter level. Low values for plasma must be interpreted with considerable reserve. It is often stated that values below 0.3 mg. of ascorbic acid per 100 ml. indicate "scurvy" ; there is however no evidence that a value of zero is not compatible with full health, and subjects on diets low in ascorbic acid reach zero values without any demonstrable ill-health. The amount in whole blood falls more slowly than that in plasma, in erythrocytes more slowly still, while the leucocyte-platelet layer of blood probably retains some ascorbic acid almost until definite signs or symptoms of deficiency appear. Typical values for a person saturated with ascorbic acid are : whole blood, 1.5 mg. per 100 ml. ; plasma, 1.7 mg. per

100 ml.; erythrocytes, 0.8 mg. per 100 ml.; leucocyte-platelet layer, 22 mg. per 100 g

Principle: A trichloroacetic acid filtrate of blood or urine is shaken with norit charcoal to clarify the solution and oxidise ascorbic acid to dehydroascorbic acid. The filtrate is then incubated with 2,4-dinitrophenylhydrazine in a mildly reducing medium produced by the addition of thiourea. The resulting bis-hydrazone is treated with strong sulphuric acid and the stable colour so formed is estimated.

Apparatus: Photoelectric (or, less suitably, Duboscq) colorimeter with 540 m μ filter

Reagents: *Trichloroacetic acid* 6 per cent solution.

Thiourea 2.5 per cent. solution in 50 per cent by volume ethyl alcohol. This solution keeps for about two months at room temperature and should readily reduce KMnO_4 .

2,4-Dinitrophenylhydrazine Dissolve 2 g in 100 ml of 9N- H_2SO_4 (3 parts of H_2O to 1 part of conc H_2SO_4) and filter. This solution, which should be filtered occasionally, keeps best at 0°C.

Norit (Activated charcoal) To 200 g. add 1 l of 10 per cent. HCl and boil. Filter with suction and wash frequently with water to remove ferric ions. Dry overnight at 110–120°C.

Sulphuric Acid, 85 per cent To 100 ml of water add 900 ml. of conc H_2SO_4 , sp gr 1.84

Method

1. Blood: Add 1 ml. of blood or plasma to 3 ml. of 6 per cent. trichloroacetic acid in a 5 ml. centrifuge tube, cork at once and shake thoroughly. After 5 min. add about 0.15 g. norit, replace the cork and shake. Stand 10 minutes, centrifuge, and filter through a 7 cm Whatman No. 1 filter paper. Place 1 ml. of filtrate in a colorimeter tube that takes a final volume of 2.5 ml., and add 1 drop of thiourea followed by 0.25 ml of the hydrazine reagent. Close the tube with a rubber bung and incubate at 37°C. After 3 hours add 1.25 ml. of 85 per cent. H_2SO_4 slowly from a burette to the tube which must be kept cooled in ice-water. After at least 30 minutes at room temperature, read in a colorimeter with a 540 m μ filter.

For the leucocyte-platelet layer, add 1 ml. of a suspension containing about 20 mg. per ml. to 2 ml. of 6 per cent. trichloroacetic acid.

2. Urine: Add 0.2 ml. of urine to 0.8 ml. of water and 2.0 ml. of 6 per cent. trichloroacetic acid. Then proceed as for blood

3. **Blanks :** For each run of estimations, two blanks should be prepared exactly as described above, one substituting water for blood or urine, and one with a sample of blood or urine and with the hydrazine reagent added after the sulphuric acid.

4. **Standards.** A calibration curve for the photoelectric colorimeter is made with standard solutions of ascorbic acid, varying in concentration from 0.25–15 μ g. per ml. of 6 per cent. trichloroacetic acid, and treated as described above.

URINARY EXCRETION

The determination of a nutrient in a 24-hour specimen of urine from a patient gives little useful information; it mainly reflects his immediate past diet. The excretion of a nutrient in the urine following parenteral or oral administration gives some indication of the state of nutrition regarding that nutrient. In field work it is in practice often difficult to collect urine for more than a few hours after administering the test dose, and "saturation tests" that involve giving daily doses and collecting samples of urine on successive days are usually impracticable; as the methods have largely been used for such work, few standards have been laid down for longer excretion periods, such as are practicable in clinical work. Various methods have been introduced for ascorbic acid (Harris and Ray, 1935; Abbasy *et al.*, 1935; Harris and Abbasy, 1937; Beck and Schorlemmer, 1938, Goldsmith and Ellinger, 1939; Kellie and Zilva, 1939; Sendroy and Miller, 1939; Basu and Ray, 1940; Harris, 1940; Richardson and Mayfield, 1940); thiamine (Harris and Leong, 1936, Najjar and Holt, 1940; Robinson *et al.*, 1940, McAlpine and Hills, 1941; Meyers, 1941); niacin (Najjar and Holt, 1941, Roberts and Najjar, 1944; Ellinger and Coulson, 1944, Coulson *et al.*, 1945), and riboflavin (Axelrod, *et al.*, 1941; Najjar and Holt, 1941). If information is required regarding all these nutrients, a combined excretion test may be performed although further work needs to be done to establish the effect of administering one nutrient upon the excretion of another: a large dose of thiamine increases the excretion of riboflavin and vice versa (Delachaux, 1941). A convenient method for studying all four nutrients is as follows, but this method can of course be used for a single nutrient (*e.g.* ascorbic acid).

Urinary Excretion Tests

Principle : The subject empties his bladder and drinks a solution containing the nutrients. The amounts of these, per Kg. of

body-weight, are : ascorbic acid, 33 mg. ; thiamine hydrochloride, 75 μ g. ; niacinamide, 1 mg. ; and riboflavin, 75 μ g. All urine passed during the next four hours, and if possible during a further three hours, is collected and analysed for the nutrients or their derivatives.

Ascorbic acid is estimated as described above. Thiamine and N¹-methylnicotinamide are estimated by adsorbing on Permutit and eluting with KCl ; then the former is converted to thiochrome by alkaline ferricyanide, and the latter to "F₂" by alkali ; both derivatives are extracted into isobutyl alcohol and estimated fluorimetrically. Riboflavin is extracted into isobutyl alcohol after treatment with potassium permanganate followed by hydrogen peroxide in presence of pyridine and acetic acid (Najjar, 1941) ; it is then estimated fluorimetrically.

Apparatus: 500 ml. brown glass-stoppered bottle, source of ultra-violet light with a Wood's glass filter or preferably a photoelectric fluorimeter

Reagents (For 100 adults) : 250 g of ascorbic acid, 550 mg. of thiamine hydrochloride, 7.5 g of niacinamide ; 550 mg. of riboflavin ; 100 g of oxalic acid, 50 g. of Permutit or Decalso ; 120 ml. of aqueous 25 per cent. KCl, 75 ml of aqueous 15 per cent. NaOH, 25 ml. of aqueous 0.25 per cent. $K_2FeC_6N_6$, 100 g of anhydrous Na_2SO_4 ; 30 ml. of pyridine + 30 ml of glacial acetic acid, 10 ml. of aqueous 4 per cent $KMnO_4$; 10 ml. of 3 per cent H_2O_2 ; 600 ml. of isobutyl alcohol.

Vitamin Test Solution. 11 g of ascorbic acid, 25 mg. of thiamine hydrochloride, 333 mg of niacinamide and 25 mg of riboflavin are dissolved in 1 litre of *N*/1000 hydrochloric acid. The solution in HCl is fairly stable

Method

The subject empties his bladder soon after waking, and urine is collected during the next 1-2 hours. He then drinks 3 ml. of the vitamin solution per Kg. of body-weight, he then has a light breakfast, low in water-soluble vitamins and without tea or coffee. All urine passed during the next four hours is stored in a brown bottle at room temperature in presence of about 400 mg. of dry oxalic acid for every 100 ml. of urine. If convenient, urine passed during the next three hours should also be collected. Obviously more complete information is obtained by collecting urine during the whole 24 hours after the test dose, as is practicable in clinical, but not usually in field work.

Estimation of Various Constituents

Thiamine and "F₂" (Johnson *et al.*, 1945). The initial steps for the absorption and elution are identical, but should be done in separate tubes.

Two ml. of fasting or $\frac{1}{2}$ ml. of loaded urine (which must be between pH 3 and 6) are pipetted into a test-tube and about 200 mg. of activated Permutit or Decalso are added. The tube is shaken for half a minute, about 8 ml. of water are added, and it is shaken again. After standing for a few minutes the supernatant liquid is discarded. The adsorbent is again washed with about 8 ml. of water, the supernatant liquid again being discarded; 0.5 ml. of the KCl solution is then added and the tube shaken gently. Henceforth the procedures for thiamine and "F₂" differ.

1. **Thiamine**: 0.1 ml. of the potassium ferricyanide solution is added, followed by 0.25 ml. of NaOH; 2 ml. of isobutyl alcohol are added and the tube is shaken vigorously for a minute. It is then centrifuged to complete the separation of the two phases. About 1 ml. of the upper layer is transferred to a small test-tube, and the fluorescence compared in ultraviolet light with standard solutions of thiochrome made from thiamine by following the steps from the start. A range from 0-200 μ g. of thiamine hydrochloride per 100 ml. is conveniently prepared, and the thiochrome in the alcoholic layer is stable in the dark at room temperature for several days. The blank correction for "F₂" can usually be neglected (Najjar and Ketron, 1944).

2. **"F₂"**: 2 ml. of isobutyl alcohol followed by 0.25 ml. of NaOH are added to the tube which is immediately shaken vigorously; the separation of the two layers is completed by centrifugation. 1 ml. of the alcohol layer is transferred to a small test-tube, and after at least five minutes the fluorescence in ultraviolet light is compared with solutions of quinine sulphate in 0.1 N-H₂SO₄, a convenient range covering 0-200 μ g. per 100 ml. The quinine sulphate standards are calibrated against solutions of N¹-methyl-nicotinamide (2 ml. of solutions containing 0.7-5 mg. per 100 ml. of 1 per cent. acetic acid, run through the whole estimation).

Riboflavin (Najjar, 1941): 0.5 ml. of urine is added to a test-tube followed by an equal volume of the mixture of acetic acid and pyridine. One drop of KMnO₄ is added and the tube is shaken gently for a minute. Two drops of H₂O₂ are then added, followed by a further drop if the permanganate is not reduced; 1.5 ml. of isobutyl alcohol are then added, and the tube is shaken vigorously

for a minute. After the tube has stood for a few minutes, a few mg. of Na_2SO_4 are added. After standing a further minute or two, 1 ml. of the clear upper layer is transferred to a small test-tube, and the fluorescence is matched in ultraviolet light with standard solutions of riboflavin (from 0-300 μg . per 100 ml.) treated exactly as was the urine

Interpretation

It must be emphasized that the technique and interpretation of urinary excretion tests are in their infancy. Each is encumbered with difficulties, both theoretical and practical; but these cannot here be discussed. At the present stage of our knowledge the clinical pathologist must by experience learn his own interpretation of the methods, controlling his estimations upon patients suspected of deficiency by comparison with those not suspect. Individual differences in absorption and in kidney threshold are large, and moderate unsaturation of the body with nutrients is common and compatible with full health. The fasting urinary excretion of a nutrient may be low but the response to the test-dose excellent, or the converse may be found, little reliance can be placed upon the fasting excretion alone. We use an enormous test-dose of ascorbic acid because many subjects in this country, apparently without detriment to their health, excrete none in their urine when some of the commoner test-doses of one-third to one-fifth of our amount are used; even with the large dose no increased excretion may be found in the next seven hours in apparently healthy subjects. The amount of nutrient given in a load test should be related to the body-weight, and the excess excreted in 4 hours or 7 hours (*i.e.* the amount excreted in, say, 4 hours less 4 times the fasting hourly excretion) should then be expressed as a percentage of the initial dose. Hourly fasting urinary excretions are usually more than 5 μg . of thiamine, 10 μg . of riboflavin and 100 μg . of N^1 -methylnicotinamide.

Conclusion

It must be emphasized once more that to assess the nutriture of a patient three procedures are essential: a clinical examination, a dietary history and a medical history. In conjunction with these, biochemical aids are very valuable, by themselves they may mislead. The valuable determinations are of haemoglobin, haematocrit (in conjunction with or replaced by an erythrocyte count), ascorbic acid in the leucocyte-platelet layer (or in whole blood,

ARBITRARY STANDARDS OF NORMALITY USED BY THE
OXFORD NUTRITION SURVEY

	Esti- mated on.	Units per 100 ml. or 100 g	Ab- normal below.	Extreme Abnorm- ality : below.
Haemoglobin				
15 yrs and over, male . . .	B	g	13.85	12.05
15 " " female . . .			12.05	10.70
7-14 yrs . . .			12.05	10.25
2-6 " . . .			11.15	9.35
0-1 yr. . .			10.25	8.45
Haematocrit				
15 yrs and over, male . . .	B	per cent	41	36
15 " " female . . .			37	32
7-14 yrs . . .			36	30
2-6 " . . .			33	28
0-1 yr. . .			30	25
Leucocrit . . .	B	per cent	0.5†	0.3†
Protein, total . . .	S	g.	6.1	5.3
Albumin . . .	S	g	4.0	3.2
Vitamin A (adults) . . .	S	I U	70	30
Carotenoids . . .	S	µg	100	50
Phosphatase (adults) . . .	S	K-A units	10*	16*
Thiamine HCl . . .	B	µg	4.5	2.0
Pyruvic acid . . .	B	mg	1.6*	2.0*
Niacin . . .	B	µg	400	200
Riboflavin . . .	B	µg	20	12
" " . . .	L	µg.	400	200
Ascorbic acid . . .	B	mg	0.50	0.10
" " . . .	L	mg.	20	10
Thiamine HCl . . .	U	µg.	5	2
N ¹ -methylnicotinamide . . .	U	µg.	100	50
Riboflavin . . .	U	µg	12	5
Ascorbic acid . . .	U	mg	0.5	0.2

B = whole blood, L = Leucocyte-platelet layer,

S = Serum, U = urine (fasting).

† Figures above 1.0 and 1.5 are also indicative of abnormality.

* Maximum normal values : phosphatase and pyruvic acid values are raised in deficiency

or, least usefully, in plasma), serum protein (preferably with fractionation), serum vitamin A and carotenoids and of serum phosphatase in infants and children. The limitations and interpretations of the other biochemical methods sketched above await elucidation by further work, and their inclusion here is justified by the hope that clinical pathologists may add to our scant knowledge in this important field.

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CHAPTER XIX

CRYSTALLINE FORM AND SOLUBILITY OF SULPHONAMIDE DERIVATIVES

SINCE the introduction and general use of the "sulphonamide" drugs the clinical pathologist has been frequently confronted with a type of crystalline urinary deposit previously unknown. The presence of this crystalline deposit is an indication that care should be exercised in the management of the patient in order to prevent dangerous complications. Urinary crystals of sulphonamide derivatives, usually the acetylated form, may be deposited either within the body (kidney, pelvis, ureter or bladder) or after the specimen of urine has been voided. In the first instance the crystalluria will be accompanied by haematuria either macro- or microscopical, and prompt and energetic therapeutic measures should be instituted to avoid, if possible, irreparable renal damage.

If the crystalline deposit appears in the absence of haematuria it generally indicates that it has formed outside the body, and suggests that generous fluid intake be given to the patient together with alkalies to prevent further concentration of the drug in the urine and deposition of crystals *in vivo* (Barnes and Kawaichi, 1943; Schwartz *et al.*, 1941).

Factors concerned in the Formation of Urinary Sulphonamide Crystals

The most important is a particular individual susceptibility on account of which only a certain percentage of the cases treated with sulphonamide drugs will show crystalluria either *in vivo* or *in vitro*. This peculiarity seems to be related to impurities contained in the urine, probably of a colloidal nature, which act as the starting point for the formation of crystals and are also responsible for the typical shape assumed: the pure synthetic drugs recrystallized from water or normal urine show formation of crystals of different type (Lehr and Antopol, 1942). Together with the individual susceptibility there appears to exist a race susceptibility: Oriental races (New Guinea, Japanese) have been found to be more liable to crystalluria after sulphonamide therapy than white people (Barnes and Kawaichi, 1943; Backhouse, 1939).

Other factors concerned in the formation of crystals in the urine

are those related to the physical properties of the solvent and of the solute, such as :

(a) Temperature and pH of the watery medium.

(b) Solubility of the sulphonamide compounds and of their acetyl-derivatives (see p. 199).

(c) Concentration of the substance in the urine : crystals will form only when the concentration in the urine has reached or passed its saturation level.

Each kind of sulphonamide drug will lead to the formation of specific crystals ; from these in many instances the type of drug employed can be identified : but the shape and size of individual crystals may vary so considerably that at times accurate identification is impossible without the aid of the polarizing microscope : with this the specific optical constants may be obtained (Prien and Frondel, 1941).

The identification from the urinary crystals of the type of drug employed in treatment is perhaps more of theoretic than practical importance : it will suffice in the presence of crystalluria and haematuria to identify the crystals as belonging to the " sulphonamide " group. For this purpose both the urine and the crystalline deposit must give a strong positive reaction when coupled with a diazotizing reagent (Aarons, 1941), and the crystals must not give a murexide reaction as urates and uric acid crystals are sometimes of similar shapes. For technical details, see p. 198.

Sulphanilamide (Fig. 9 A) : colourless, long, thin slates with two sides parallel : one end terminating in a point or broken off irregularly, and the other ending squarely with angles of 90° . Also needle-like crystals, often in bundles or sheaves. It occurs rarely in the urine on account of the high solubility of the pure drug and of its acetyl-derivative.

Sulphapyridine (Fig. 9 B) . very variable in size and shape. Most characteristic are the rhomboid, petal- or boat-shaped forms, thin, colourless, transparent. Crystals are frequently bounded by curved or irregular surfaces and have been often described as lenticular, whet-stones or arrow-heads. In typical crystals the acute angle is of 77° . Occasionally it takes the form of aggregates of needle-like crystals (spherulites) in the shape of bow-ties, cross- or star-like structures, etc. Crystals are fairly frequently encountered in urinary sediments as the pure drug and its acetyl-derivative are amongst the less soluble of the group.

Sulphathiazole (Fig. 9 C) : typical crystals, rarely found, are hexagonal, flattened on a pair of faces, yellowish-brown in colour,

with the two opposite angles of 136° . It is more frequently found in the form of spherulites in the shape of bow-ties, rosettes, spherical spiny burrs, etc. These aggregations of needle-like crystals always

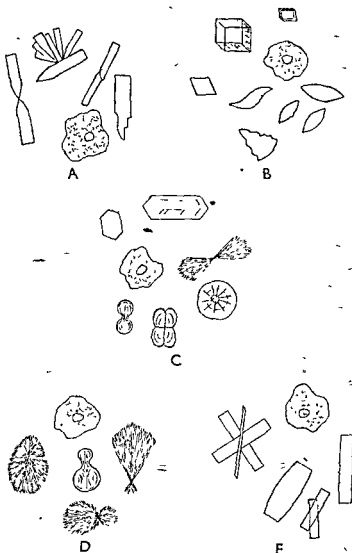


Fig 9 Sulphonamide Crystals

form around a central point and therefore appear symmetrical. The acetyl-derivative especially in alkaline urine is more soluble than that of sulphapyridine and crystals are not so frequently encountered.

Sulphadiazine (Fig. 9 D) : generally in the form of aggregates of needle-like or long petal-shaped crystals, yellowish-green in colour, similar to those of sulphathiazole : but all aggregations occur around an eccentric point and all structures are therefore asymmetrical. Crystals of the acetyl-derivative are not very frequently seen as its solubility is greater than that of sulphapyridine or sulphathiazole or of free sulphadiazine. The pure drug, however, is the least soluble of all, and crystals of free sulphadiazine have been observed (Lehr and Antopol, 1942) in the form of globular aggregates of needle-like crystals (chestnut burr).

Sulphaguanidine (Fig. 9 E) : long, thin, colourless slates with the two long sides parallel or slightly bulging in the centre (barrel-shaped) : both ends terminate squarely. Frequently conglomerates in cross- or star-like structures. Sulphaguanidine is reputed to be poorly absorbed : however the results of Stanier and Stapleton (1944) show that it is as readily absorbed as most of the sulphonamides. Crystals have been observed generally in cases suffering from severe lesions of the intestinal mucosa, such as ulcerative colitis.

Identification of Urinary Crystals

Wash the crystalline deposit two or three times with cold distilled water. The crystals will be found to settle rapidly to the bottom of the test tube and to separate readily from other material such as epithelial cells, red cells, etc. Hydrolyze the washed sediment with 4*N*-hydrochloric acid, add a few drops of 0.5 per cent. sodium nitrite and finally 5-10 drops of 1 per cent. dimethyl- α -naphthylamine in alcohol (or 0.5 per cent. *N*-(1-naphthyl)-ethylene-diamine hydrochloride in water). In the presence of crystals of sulphonamide derivatives a pink to red colour will develop readily.

The urine is treated first with a few drops of 20 per cent trichloroacetic acid (or 15-20 per cent. *p*-toluene-sulphonic acid, then coupled with sodium nitrite and naphthylamine as for the crystals, when a pink to red colour will rapidly appear in the presence of sulphonamide derivatives.

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Solubilities of Sulphonamides

The solubility of the sulphonamides and their acetyl-derivatives are of importance, as one of the factors responsible for the formation of crystalline deposits in the urine.

The solubilities of some of the common sulphonamides in buffer solutions at various pH 's are given in the following table, much of which is taken from the paper of Gilligan *et al.* (1943).

The figures for sulphanilamide were determined in the author's laboratory.

Name of Sulphonamide	Solubility of Base			Solubility of Acetyl-derivatives		
pH	5.5	6.5	7.5	5.5	6.5	7.5
Sulphanilamide	1440	1440	1450		534 ²	
Sulphapyridine	61	61	62	33	34	37
Sulphathiazole	98	108	235	7	9	28
Sulphadiazine	13	28	200	20	75	512
Sulphamethyldiazine (Sulphamerizine)	35	45	170	38	57	272
Sulphadimethyldiazine ¹ (Sulphamethazine, Sulphamezathine)	69	76	140	90	107	240 ¹

¹ These figures have not been available since (cf Gilligan and Plummer, 1943).

² From M R C Monograph pH not stated

It will be noted that the solubilities of sulphanilamide and sulphapyridine are very much less affected by increase of pH over the range of pH 5.5-7.5 than the others, which show a very marked increase over this range. This is because, as shown by Fox and Rose (1942) the sulphonamides act as very weak acids, with pK 's of 10.5 for sulphanilamide, and 8.5 for sulphapyridine, 6.8 and 6.4 for sulphathiazole and sulphadiazine. The proportions of drug ionized at pH 7.5 calculated from these figures are 0.03 per

cent. and 3.4 per cent. for sulphanilamide and sulphapyridine, but 61.6 per cent. and 80 per cent. for sulphathiazole and sulphadiazine. From these figures, it will be seen that no marked increase in the solubility of sulphanilamide or sulphapyridine itself is to be expected by changes with the physiological range.

The figures given in the table refer to solubility in aqueous buffer solutions, which approximate closely to the solubilities in urine at the same pH . In serum or other protein containing fluids, an appreciable fraction of the drug is combined with protein (Davis, 1943) and the solubility is much greater than in aqueous solutions. The portion of drug bound to protein is not dialyzable, and this binding accounts for the fact that whereas the concentration of sulphanilamide is approximately the same in serum and cerebrospinal fluid, the cerebrospinal fluid/serum ratio is very much less than 1 for the other drugs.

The distribution of drug between cells and serum is discussed by Heinemann (1943). Here, too, the change in the case of sulphanilamide itself is much less than that shown by the other sulphonamide compounds.

Finally, it is noteworthy that the acetyl compounds of the diazines, in contradistinction to the other compounds, are more soluble at all pH 's than the corresponding free bases.

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Estimation of Sulphonamides in Blood

(See p. 220.)

CHAPTER XX

PHOTOELECTRIC COLORIMETERS

DURING the last few years, the Duboscq type of colorimeter has been steadily losing ground in popularity to its recent competitor the photoelectric colorimeter. The chief advantages of photoelectric instruments are (a) the elimination of personal error and eyestrain, (b) greater speed in making readings, and (c) greater accuracy. Moreover, in those estimations where it is necessary to allow for a blank colour from reagents, a "blank" can be substituted for water in making the readings; this is not possible with the ordinary Duboscq colorimeter. Although some of the commercial photoelectric colorimeters are expensive, a reliable instrument may be assembled at less than the cost of a good visual colorimeter.

PRINCIPLES OF PHOTOELECTRIC COLORIMETRY

Photoelectric colorimeters (or, better, photometers) are instruments which measure the amount of light absorbed by a coloured solution under given conditions. In general, light is passed through the test solution, and the emergent light directed on to a photo cell, where the degree of absorption is measured by one of the procedures to be described.

Photo Cells: Photo cells are of two kinds (1) the so-called barrier-layer type which is self-energizing and produces a voltage dependent upon the amount of light which falls upon it, and (2) cells of the potassium-silver oxide type which depend on a change of electrical resistance when light falls upon them. Because of its simplicity, reliability and long life, the barrier-layer photo cell is preferable and remarks will be confined to this type. It consists essentially of a thin layer of selenium affixed to a heavy metal base; this is covered by a thin transparent metal layer, varnished except for a portion mechanically strengthened to form a collecting ring. When light, penetrating the thin metal layer, falls on to the selenium, electrons are released, which penetrate a hypothetical barrier layer, between the selenium and transparent metal layer, forming a negative charge on the latter. Thus under the action of light we have a cell whose negative pole is the metal collecting ring, and positive pole the metal base.

General Considerations: When light passes through a coloured solution, the amount of light absorbed is proportional to the number of absorbing molecules in its path, that is, to the concentration of the coloured substance. This is usually expressed as Beer's law, which states that the ratio of the amount of light absorbed by two solutions of a coloured substance is equal to the ratio of their concentrations, provided that these concentrations are not widely different.

In many biochemical estimations, particularly if monochromatic light of the optimum wave length be used, the light absorption is proportional to the concentration of coloured substance over a wide range and consequently less emphasis need be placed on the importance of the concentrations of the standard and test being close together. In some instances, proportionality is so good that only one standard need be used, no matter what the concentration of the test, but in general, it is advisable to study each method from this point of view by preparing and reading a series of standards. If this be done with several colour filters, it will be found that one of them will give better proportionality than the others: this is the filter of choice.

Some workers use such a calibration curve to calculate their results, but it is recommended that a standard (or more than one where indicated) be prepared and read at the same time as the tests. This serves as a check on the procedure, since any change from the usual reading will draw attention to error.

While the concentration of the coloured substance is directly proportional to the amount of light absorbed it bears a logarithmic relationship to the ratio of the incident and emergent light intensities, as shown in the theoretical section which follows.

Theoretical: When a beam of light passes through an absorbing medium, it can be shown that

$$E.C.l = -\log \frac{I_1}{I_0}$$

where I_0 and I_1 are the intensities of the incident and emergent light respectively,

l is the thickness in cm. of the coloured solution through which the light has passed,

C is the concentration of the coloured substance in mg. per ml.,

and E is a constant called the specific extinction coefficient.

The expression $-\log \frac{I_1}{I_0}$ is also known as the optical density.

Photoelectric Colorimeter

Photoelectric colorimeters may be divided into two classes; those which require one photocell and those needing two cells.

"One cell" Instruments: A simple and widely used instrument is the type described by King (1942) in which light from a flash light actuated by an accumulator passes, by means of a path cut in a wooden block, through a test-tube on to a photo-cell. An adjustable metal bolt is also fixed in a vertical hole across the light path to enable the amount of light reaching the test-tube to be controlled. A groove at the front of the wooden block permits a suitable light filter to be inserted. A sensitive galvanometer, fitted with a reciprocal logarithmic scale is connected across the photo-cell to measure the strength of the current produced under the action of light.

In use, the test-tube filled with water is inserted and the bolt adjusted until the galvanometer reads its maximum deflection (0 on the reciprocal logarithmic scale). The coloured solution is now placed in a carefully matched test tube, and inserted in the instrument. This coloured solution will absorb light, so that less will fall on the photo-cell, a lower voltage will be produced and consequently the galvanometer needle will move nearer its resting position, that is nearer infinity on the logarithmic scale. The darker the test solution, the greater will be the amount of light it absorbs, and the greater will be the reading on the logarithmic scale. It is advisable to compare with a standard read in a similar manner when subject to the general considerations given above.

Concentration of "test"

$$= \frac{\text{reading of test}}{\text{reading of standard}} \times \text{concentration of standard}$$

"Two-cell" instruments. The Spekker Absorptiometer (Hilger 1936) may be taken as an example of a "two-cell" instrument. Two matched photo-cells connected in opposition to a galvanometer are mounted one on either side of a central light source so that any variation in illumination due to fluctuation of current affects the two cells equally. On each side, light "passes" through a colour filter, an adjustable diaphragm, and a glass cell before reaching the photo-cell. The diaphragm on the right hand side is fitted with a scale calibrated in such a way that if R is the reading corresponding to a degree of opening such that the amount of light transmitted is $1/a$ of that admitted when the aperture is

fully open then $R = \log a$. In use, the right side scale is set at zero, and with the test solution on this side, the galvanometer, which acts as a null-point instrument is brought to zero by adjustment of the aperture on the opposite side. Water or a blank solution is now substituted for the test solution. Less light will be absorbed on this side and, consequently, the galvanometer needle will move away from the zero, but will be brought back by adjustment of the calibrated scale on the right hand side. The scale reading at this point will be a measure of the amount of light absorbed by the coloured solution. A standard solution may also be read in a similar manner. Then,

Concentration of "test"

$$= \frac{\text{reading of test}}{\text{reading of standard}} \times \text{concentration of standard}$$

Choice of Photoelectric Colorimeter: "Two-cell" instruments have the theoretical advantage that changes in intensity of illumination have equal effects on the two cells, and since these are connected to the galvanometer in opposition, such changes have no effect on the galvanometer reading. "Single-cell" instruments, on the other hand, cannot be used in connection with the mains, except with a constant voltage transformer. In practice, however, it is found that this difficulty is not a real one, and should not be a factor in the choice of an instrument. The current supplied by the small inexpensive type of radio accumulator is perfectly constant, or the inequalities in the mains supply may be "ironed out" by the use of a constant voltage transformer.

The advantage of this model is that it is inexpensive, may be assembled by any one with a rudimentary knowledge of carpentry, and is very simple and rapid in use. Bell and Guthman (1943) have described another type of "one-celled" instrument, in which readings are taken by movement of the source of light along a calibrated scale. This arrangement, however, possesses no advantage over the simpler King system.

Construction of a Simple One-cell Instrument: The construction and assembly of the instrument are illustrated in the diagram. An ordinary surgical head-lamp (Ever-ready) furnishes the source of light (E). The bulb is activated from an accumulator, which ensures that a steady, non-fluctuating light is obtained. Immediately in front of the lamp is mounted a block of wood (well-seasoned teak or oak $7.5 \times 7.5 \times 5$ cm.) which has been bored vertically to fit a 1.5 cm. test-tube (A) and a 1.25×5 cm. metal

bolt (B) whose nut is countersunk in the block. The bolt, which should fit its hole tightly, is used to vary the size of the slot through which the light passes. The slot is made by boring a 1.25 cm. hole from front to back, cutting through the vertical holes, and then sawing up from the bottom of the block to make the slot 1.25 cm. wide by 3.75 cm. high. At the front of the block a groove is made 2.5 cm. wide and 0.3 cm. deep to carry a light filter (D). A similar groove 2.2 cm. wide is made at the back of the block to fit the photoelectric cell (C). The block is mounted on a 7.5×12.5 cm. piece of wood, and the 1.5 cm. hole is extended 0.6 cm. into the second piece so that the end of the test-tube drops below the bottom of the slot.

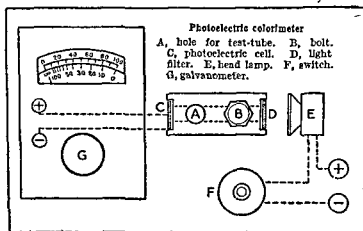


FIG. 10.—Direct-reading Photoelectric Colorimeter.

The photoelectric cell is of the selenium type, 22×40 mm. The "EEL" electroselenium cell supplied by Messrs. Arthur E. Evans of Bishop's Stortford, Herts., has proved satisfactory. A copper wire is fitted on the surface of the rear groove in suitable position to make contact with the exposed strip of selenium which lies near the edges of the front surface of the cell. The wire is connected to an ordinary electric terminal screwed into the back of the block. A lead from this front-surface terminal connects with the negative terminal of the galvanometer. A second terminal carries a spring clip which holds the photoelectric cell in place and makes contact with its back surface. The back-surface terminal is connected to the positive terminal of the galvanometer. Both the sensitivity and accuracy of colour measurement are increased by the use of light filters. As in visual photometry, the measurement

of colour intensity by photoelectric means is best carried out in light restricted to parts of the spectrum whose light is maximally absorbed by the coloured solution under investigation. This condition is approximated with the filters recommended.

The galvanometer for use with this instrument should be of about 1,000 ohms resistance and should have a maximum deflection corresponding to about 10 microamperes. The pointer type and the reflector type have been found equally satisfactory.

The most convenient galvanometer scale is a logarithmic one on which the zero point corresponds to maximum deflection. The galvanometer reading on the logarithmic scale is then directly proportional to the light absorbed. A set of coloured solutions containing varying amounts of the same pigment will give readings which are directly proportional to the concentration.

Operation of the Instrument

The galvanometer is levelled, the suspension is released and the "spot" or pointer adjusted to the ∞ mark at the left of logarithmic scale (0 on the linear scale) by turning the suspension knob. The light is now switched on. The bolt is turned up or down to regulate the amount of light reaching the photoelectric cell, until the needle is at 0. It should remain at this point and will do so after the first few minutes if the electric supply is taken from a properly charged accumulator.

A set of test-tubes is selected which will fit the hole snugly but easily. They should all be of similar glass and free from obvious streaks and scratches. A test-tube is half filled with water, wiped with a towel to remove any stains and placed in the hole. The bolt is turned until the needle is at 0. A scratch is made on the side of the test tube opposite a mark on the block to indicate that the tube should always be used in that position. If on turning the tube in the hole more than a scarcely perceptible movement of the needle takes place the tube should be discarded since it contains flaws in the glass which are interfering with the passage of light. The other tubes are tested similarly and a mark made on each to indicate in which position it should be inserted to ensure equal light transmission. These tubes may now be used interchangeably. The reactions leading to the production of the coloured solutions may be carried out in them so that no transference of the solution is necessary when it is read in the colorimeter. A good quality of fairly thin-walled test-tubes should be used. (For more accurate work the very thin-walled and uniform test-tubes of the type used

in the Lovibond comparator are useful.) The most convenient size is 1.5×12.5 cm. It will be found useful to mark them with a file to indicate volumes of 5 ml., 10 ml. and 15 ml.

The appropriate light filter for a coloured solution is next tested for. This will usually be the filter which gives the highest reading, *i.e.* absorbs most light, and which gives most nearly a direct proportionality in reading with different concentrations of pigment. Generally speaking its colour will be "complementary" to that of the solution, for a blue or green solution a red filter, for a yellow solution a blue filter, and for a red solution a green filter is needed. The selection of the filter is made as follows.

With the filter and test tube in place, the galvanometer needle is adjusted to 0 by turning the bolt. The test tube of water is removed and another containing the colour standard is inserted. The galvanometer needle is allowed to come to rest and the reading is recorded. On replacing the tube of water the needle should return to 0. If it fails to do so, the light is again adjusted by turning the bolt and the reading for the standard is again taken. This operation is now repeated with a second standard of double the strength of the first. The reading of the second standard should be twice that of the first. If this condition is not fulfilled, another light filter is tried. Sometimes the curve relating concentration to the colorimetric reading is a straight line *not* passing through the origin. In this case proportionality will not be good. This is almost certainly due to the presence of a "blank" colour, *i.e.* a colour arising from the reagents which have been used to produce the colour by reaction with the standard substance. A "blank" consisting of the reagents and water, with no added standard, is prepared and substituted for the water in the 0 adjustment. Proportionality should now be obtained with the appropriate filter.

The micromethods of analysis for blood devised by King *et al.* (1937, 1942) which are described in Chapter XXI have been readily adjusted for use with the photoelectric instrument. Very few changes have been necessary: they are given in the table, together with the light filter recommended for each method. As in visual colorimetry the reading of the standard which is nearest the test should be used in the calculation.

Photoelectric colorimeters can be supplied by the following firms: Evans Electro selenium Co., Bishop's Stortford, Herts.; A. Gallenkamp & Co., Sun St., London, E.C.2; Gambrell Bros. & Co., Ltd., 307, Merton Rd., London, S.W.18; Adam Hilger, Ltd., 98, St. Pancras Way, London, N.W.1.

Adaptations of Micro-Chemical Methods described in Chapter XXI for use with the Photoelectric Colorimeter

Substance	Changes necessary.	Light filter.
Urea and N.P.N	Use a blank of 7 ml H_2O + 1 ml. Nessler's for 0 adjustment of galvanometer.	Chance OB. 2 or Ilford minus red.
Uric Acid	Dilute tests and standard to 5 ml. with water (very high ones to 10).	Chance OR. 2 red, or Ilford tricolour red
Creatinine	Dilute tests and standard to 5 ml. with water. Use a blank consisting of water + 0.5 ml. of alkaline picrate.	Chance OB. 2 or Ilford minus red.
Phosphate	Dilute to 5 ml. with $N H_2SO_4$.	Chance OR. 2 red, or Ilford tricolour red
Cholesterol	No change.	Chance OR. 2 red or Ilford minus green.
Glucose	Colorimetric method of Haslewood and Strookman. Dilute with water to 10 ml, or 15 or 20 ml. for very high sugars	Chance OR. 2 or Ilford tricolour red.
Sulphonamides	No change.	Chance OGr. 1 green or Ilford tricolour green
Proteins	Use a blank consisting of 0.2 ml 50 per cent. H_2SO_4 , 5 ml. water and 3 ml Nessler's.	Chance OB 2 or Ilford minus red.
Sodium	1 ml of coloured solution diluted to 10 ml with 0.5 per cent. acetic acid.	Chance OB 2 or Ilford minus red.
Phosphatase	Dilute to 10 ml. with water. (High phosphatase further diluted.)	Chance OR. 2 red, or Ilford tricolour red
Potassium	No change.	Chance OR. 2 red or Ilford tricolour red
Haemoglobin	Alkaline haematin method. 0.1 ml. blood in 10 ml $N/10$ NaOH. Standard ($\equiv 14.8$ g. Hb/100 ml) 25 mg. pure cryst. haemin (8.57 per cent Fe) in 1 lit. $N/10$ NaOH.	Chance OGr. 1 green or Ilford tricolour green

¹ Test tubes for use in the photoelectric colorimeter should be marked with a file or diamond to indicate the levels at which they contain 5, 10 and 15 m.

E. J. KING.
G. E. DELORY.

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CHAPTER XXI

MICROMETHODS OF BLOOD ANALYSIS

Notes on Collection of Blood for Analysis

THE majority of methods for blood analysis require 1 ml. or more for a single determination and are adequately described in the standard textbooks of clinical chemistry. For many purposes accurate methods requiring smaller amounts have great advantages and a number of these are described in this chapter. The majority have been worked out by King and his colleagues.

For full value to be made of biochemical analyses it is essential that blood should be taken under the proper conditions and handled properly prior to analysis. There is no value in having analytical methods accurate to say 1 per cent. if the previous handling of the blood leads to a variation of several per cent. in a certain constituent. For this reason a general account is given of the proper methods of taking blood for various tests.

For the micromethods on whole blood described below, the blood is best obtained by finger prick and taken up into a capillary pipette calibrated "to contain" and washed straight from this pipette into isotonic sodium sulphate or other solution described in the various methods, but if blood is being sent a distance this is impracticable and venous blood is required. The following notes may be helpful in producing satisfactory blood samples.

Choice of Whole Blood or Serum or Plasma: Certain blood constituents such as urea are evenly distributed between cell and serum water, and for these it is immaterial which is used for analysis. In others such as glucose, the distribution is somewhat uneven, but custom and convenience has led to most determinations being made on whole blood. The distribution of sulphonamides between cells and corpuscles is grossly unequal and varies from one sulphonamide to another. Sulphanilamide, for instance, is at a much higher concentration in the cells and sulphathiazole in the plasma, and rationally sulphonamide determinations should not be carried out on whole blood; nevertheless, most of the previous work has been done on whole blood and for convenience whole blood will continue to be used, although the worker should realize this point in interpreting results, particularly with a new sulphonamide. The majority of determinations of the non-protein-nitrogenous constituents are usually carried out on whole blood.

In contrast to the above, analyses of whole blood for any of the electrolytes are for ordinary purposes useless. Chlorine is the least unevenly distributed electrolyte, but even here the plasma contains nearly twice as much chlorine as the cells, and variations in the cell/plasma ratio, as in anaemia, lead to grosser alterations in whole blood chloride levels than is met with in the majority of diseases. Early work on whole blood has led to the rather widespread and erroneous belief that blood in nephritis has a higher chlorine content than normal; chloride retention in nephritis is uncommon, but anaemia, causing a higher proportion of chloride-rich plasma in a sample of whole blood leads to the high whole blood chloride levels. Of the other electrolytes, the sodium content of the plasma is high, of the cells very low, while potassium is high in cells and low in plasma; calcium does not occur in cells at all. On account of this unequal distribution, plasma or serum is necessarily used for all electrolyte analyses.

Anticoagulants: Various substances are used to prevent blood clotting. The commonest are sodium or potassium oxalates, potassium fluoride (for blood sugar), sodium citrate, lithium oxalate and heparin.

For the prevention of clotting where analyses are to be made on whole blood an oxalate (in reasonable quantity) is usually used but causes osmotic changes in the blood with a fall in the haematocrit reading, and for that reason a mixture of potassium and ammonium oxalates (6 mg. potassium oxalate and 4 mg. ammonium oxalate for 5 ml. blood) is frequently used; it causes no osmotic changes in the blood but is not usually suitable for urea or non-protein-nitrogen determination as the ammonium salt causes a very high blank. Fluoride is often used when taking blood for sugar analysis, if analysis is not to be performed immediately, as it prevents glycolysis which may go on rapidly in warm weather in plain oxalated blood. Fluoride blood cannot be used for urea determination by the urease method, as the fluoride is inhibitory to the enzyme. In the micromethod described here (p. 214) copper sulphate is used to prevent glycolysis.

Heparin (0.1 mg. per ml.) is the most satisfactory all-round anticoagulant, but is not widely used on account of expense and the difficulties of supply. Owing to the osmotic changes produced by the other anticoagulants, heparin is the only one reasonably satisfactory for use when any of the electrolytes are to be determined. For simplicity, serum is generally used for all electrolyte determinations. The changes produced by the oxalates are most obviously

shown by determining the protein content of serum and oxalate plasma from the same blood. In spite of the added content of fibrinogen in the plasma, the total plasma protein is frequently found lower than the serum protein, owing to dilution of plasma by water abstracted from the cells through the osmotic activity of the salts.

Avoidance of Haemolysis : Haemolysis interferes with many tests, not only biochemical, and must be avoided. The following points will aid in avoiding haemolysis. The syringe is preferably dry sterilized ; it may be lubricated with liquid paraffin. If for any reason a wet syringe has to be employed, it must be rinsed out thoroughly with normal saline, and not with water ; ether should not be used for drying, as traces very easily produce haemolysis. The blood should be delivered into the container slowly, after the needle has been removed ; forcible squirting through the needle causes gross haemolysis. If for any special reason oxalate plasma is required, one drop of 20 per cent. potassium oxalate for 10 ml. of blood is better than dry oxalate ; the very slight dilution error (about 0.5 per cent.) causes less harm than possible haemolysis through the use of the dry salt.

Avoiding Loss of Blood Gases : For estimation of sodium, chlorine and bicarbonate, escape of carbon dioxide from the blood should be avoided. If CO_2 escapes, water and chlorine ions pass from cells to plasma and the composition of the serum when separated differs from that which it had in the circulation. For accurate work "true" serum can be obtained without difficulty by the following technique.

About 1 inch of liquid paraffin is placed in a small screw-capped bottle ("Universal container"). Liquid paraffin is also placed in a well-fitting syringe so as to eliminate all dead-space in the nozzle and needle. The arm is compressed by a nurse to make the veins prominent, the needle is inserted and the blood withdrawn. Pressure must not be applied for more than a few seconds before the needle is inserted, and a tourniquet should not be used, as stasis causes changes in the CO_2 content of the blood and a rise in haemoglobin and protein content. After the blood has been obtained, the point of the needle is passed below the liquid paraffin in the container and the blood gently and slowly expelled under the paraffin. The bottle is then filled right to the top with paraffin and the screw cap replaced. The blood can then be allowed to clot, centrifuged after the clot has been loosened with a very fine glass rod, and the serum pipetted off with a fine Pasteur pipette.

It is useless merely to cover the blood with a layer of paraffin in an open tube; CO_2 rapidly diffuses through the paraffin and is lost.

If blood is not taken with these precautions, errors in the sodium, chlorine and bicarbonate levels occur and it is necessary to appreciate their magnitude. The sodium and chlorine levels are usually not more than 4 per cent. low, but the bicarbonate (even if resaturation of the serum with alveolar air is carried out) may be low by as much as 10 per cent. Figures with this degree of inaccuracy may be sufficient to diagnose a severe acidosis, but are undesirable in accurate scientific work.

For calcium, potassium and phosphate determinations these precautions are not necessary, but it is essential to separate serum within 2-3 hours at most for estimation of potassium. If serum is left in contact with clot for longer than this, potassium begins to diffuse out and serum obtained after standing overnight in contact with clot may have a potassium content 100 per cent. higher than it had originally: as cells contain some 20 times as much potassium as the serum, it is obvious that scrupulous avoidance of haemolysis is essential in all work on serum potassium. The cells also contain large quantities of organic phosphoric esters, and these gradually break down on standing to produce erroneously high values for inorganic phosphate. For phosphate determinations, either on whole blood or serum or plasma, deproteinization must be done within 2 hours, to avoid abnormally high values through hydrolysis of phosphoric esters.

MICROCHEMICAL METHODS

The methods described in the following section, using in most cases 0.2 ml. of blood or plasma for determination were published by King and his colleagues in 1937 and 1942. Modifications in the colorimetric determinations required if a photoelectric colorimeter is used are described on p. 208. The spectral filters given on p. 208 can with advantage be used even if an ordinary colorimeter is used, when sharper readings will be obtained

References

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1. GLUCOSE

Principle: Harding's modification of the Schaffer-Hartmann method, using unclaked blood, gives true sugar values. The non-sugar reducing substances (chiefly glutathione) in the corpuscles are excluded by taking the blood into isotonic sodium sulphate solution in which the corpuscles remain intact. Normal fasting blood sugar values of 65-90 mg. per 100 ml. are found.

Reagents required:

Isotonic sodium sulphate: 30 g. of crystalline sodium sulphate ($\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$) are dissolved in water and made up to 1 litre

7 per cent copper sulphate

10 per cent sodium tungstate

Copper Reagent Solution A 13 g copper sulphate crystals are dissolved in water and volume made up to 1 litre. Solution B 24 g. Rochelle salt (sodium potassium tartrate), 40 g. anhydrous sodium carbonate, 50 g sodium bicarbonate, 36.8 g potassium oxalate and exactly 1.4 g potassium iodate are dissolved separately in the minimum quantities of water at room temperature. The solutions are then mixed and volume made up to 1 litre.

The "copper reagent" is a freshly made mixture of exactly equal volumes of solutions A and B

(Note—Only purest analytical reagents should be used in making the above solutions. When preparing a fresh copper reagent it is desirable to check it against a solution of pure glucose.)

Method: 0.2 ml of blood is added to 3.2 ml. of the isotonic sodium sulphate solution and 0.3 ml. of 7 per cent. copper sulphate. After 4 minutes standing, to allow diffusion from the cells, 0.3 ml. of 10 per cent sodium tungstate is added.¹ The mixture is shaken and then centrifuged.

Two ml of supernatant are treated with 2 ml. of fresh copper reagent in a wide ($\frac{3}{4}$ inch) test-tube. A blank is prepared from 2 ml. water and 2 ml. reagent. Both tubes, stoppered lightly with cotton wool are placed in a boiling water bath for exactly 10 minutes. They are then cooled at once under the tap. To each is added 2 ml. of 1 per cent. potassium iodide and 2 ml. of *N*-sulphuric acid. After standing 1 minute the contents of each tube are titrated with *N*/200 sodium thiosulphate. One per cent. soluble starch (made up in water or, better, in saturated phenol red solution) is used as

¹ If the sugar determination cannot be made immediately it is advisable to keep the blood in the isotonic sodium sulphate containing copper sulphate to prevent glycolysis.

indicator. The titration figure of the test solution is subtracted from that of the "blank."

Calculation: 1 ml. N/200 thiosulphate \equiv 0.116 mg. glucose. Therefore (ml. of thiosulphate) i.e. (difference between the "blank" and "test" titrations) \times 116 = mg. of glucose/100 ml. blood.

If the blood sugar value thus obtained is greater than 400mg./100 ml., the determination should be repeated, using as test solution a mixture of 1 ml. of filtrate and 1 ml. of water. The result then obtained is multiplied by 2.

2. UREA

Principle: The sample of blood is digested with urease, and the urea thus converted into ammonia. After removal of proteins, the colour produced by the ammonia with Nessler's reagent is compared colorimetrically with the colour produced under the same conditions with a standard ammonium chloride solution.

By using *unlaked* blood, cloudiness due to precipitation of glutathione and ergothioneine from the red cells by Nessler's reagent is avoided, and arginase of red cells does not liberate ammonia from the arginine present in many commercial urease preparations.

Reagents required:

Nessler's reagent As described in Peters and Van Slyke's "Quantitative Clinical Chemistry," Baltimore, 1932, vol. II, p. 532, and in Beaumont and Dodds' "Recent Advances in Medicine," 11th ed., London (1943), p. 360

Standard ammonium chloride solution (containing 0.01 mg. of nitrogen per ml.): 153 mg. of pure ammonium chloride are weighed out and dissolved in water. The volume is made up to 100 ml. 25 ml. of this solution with 10 ml. of N-sulphuric acid are diluted to 1 litre with distilled water.

Isotonic sodium sulphate As used for blood sugar ✓

Ten per cent zinc sulphate 10 g. of crystalline zinc sulphate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) are dissolved in water and made to 100 ml.

Method: 0.2 ml. of blood is added to a centrifuge tube containing 3.2 ml. of isotonic sodium sulphate solution.

A "knife point" (about 20 mg.) of Jack Bean meal is added, and the whole stoppered with a rubber bung, mixed, and incubated at 37°C. for 20 minutes. 0.3 ml. of zinc sulphate solution and 0.3 ml. of 0.5 N-sodium hydroxide are added to precipitate the proteins, and the mixture is centrifuged. 2 ml. of the supernatant fluid represent 0.1 ml. of blood.

Two ml. of the clear supernatant are treated with 5 ml. of water and 1 ml. of Nessler's reagent. The solution is compared in a colorimeter with a "high" or "low" standard made up with 2 ml. or 5 ml. of the standard ammonium chloride solution (0.01 mg. of nitrogen per ml.), 5 ml. or 2 ml. respectively of water, and 1 ml. of Nessler's reagent. The colorimetric comparison is facilitated by the use of a Klett violet light filter (Messrs. Chas. Hearson).

Calculation :

(1) "Low" standard :

$$\text{Urea (mg. per 100 ml.)} = \frac{\text{Reading of test}}{\text{Reading of standard}} \times 42.8$$

(2) "High" standard :

$$\text{Urea (mg. per 100 ml.)} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 107$$

N.B.—1 mg. of nitrogen \equiv 2.14 mg. of urea.

3. NON-PROTEIN NITROGEN

Principle : The proteins of laked blood or plasma are precipitated by tungstic acid. The filtrate is digested with sulphuric acid and the nitrogen estimated colorimetrically as ammonia with Nessler's solution.

Reagents required :

Nessler's reagent, and *ammonium chloride standard* as described for urea

Sodium tungstate : 10 per cent in water.

$\frac{2}{3}$ N-Sulphuric acid

30 per cent sulphuric acid 30 ml concentrated acid are allowed to run slowly and with shaking into about 60 ml of distilled water in a 100 ml volumetric flask. The mixture is cooled to room temperature, made to the mark, and mixed

Method : 0.2 ml. of blood (or plasma) is pipetted into 3.2 ml. of water or isotonic sodium sulphate solution. Proteins are precipitated by the addition of 0.3 ml. of 10 per cent. sodium tungstate and 0.3 ml. $\frac{2}{3}$ N-sulphuric acid. The tube is stoppered and thoroughly shaken. After five minutes the mixture is filtered.

One ml. of the filtrate (\equiv 0.05 ml. of blood or plasma) is evaporated in a test tube with 0.5 ml of 30 per cent. sulphuric acid until the liquid turns dark and white acid fumes are evolved. The cooled liquid is then treated with 1 drop of hydrogen peroxide (99-100 vols.), to destroy any coloured products, and boiled for 4 minutes.

To the cooled solution are now added 5 ml. of water and 3 ml. of Nessler's solution. The colour produced is compared in the colorimeter with the "low" or "high" standard used in the determination of blood urea.

Calculation.

(1) "Low" standard :

$$\text{Non-Protein Nitrogen (mg. per 100 ml.)} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 40$$

(2) "High" standard :

$$\text{Non-Protein Nitrogen (mg. per 100 ml.)} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 100$$

4. URIC ACID

Principle : Blood in isotonic sodium sulphate solution is treated with a phosphotungstic acid reagent. This precipitates the proteins, and on addition of sodium cyanide to the supernatant liquid, the excess of phosphotungstic acid reagent produces a blue colour with uric acid present. The colour is compared with that given by a standard solution of uric acid. The blood must not be laked, as interfering substances, such as glutathione and ergothionine, would be liberated from the cells.

Reagents required :

Isotonic sodium sulphate as under blood urea.

Sodium cyanide-urea reagent : 5 g of sodium cyanide and 20 g. of urea are dissolved in water, and the volume made to 100 ml. The urea prevents clouding during the determination.

Folin's (1934) uric acid reagent : (1) Preparation of molybdate-free sodium tungstate. a solution of 250 g of sodium tungstate in 500 ml. of water is treated with 5 N-hydrochloric acid until neutral to litmus paper. The solution is saturated with hydrogen sulphide, and allowed to stand 24 hours. It is then treated with 400 ml of absolute alcohol, added gradually with constant shaking. The mixture, after standing for a further 24 hours, is filtered, and the precipitate washed with 50 per cent. alcohol and dissolved in 375 ml. of water. 0.5 ml of bromine is added, and the mixture boiled gently until the excess bromine is dispelled. Sodium hydroxide solution (40 g per 100 ml) is now added to the hot solution until the latter is alkaline to phenolphthalein. The cooled solution, filtered if necessary, is treated with 200 ml of absolute alcohol, and allowed to stand for 24 hours. The white crystals are filtered off and dried in a desiccator.

(2) Preparation of reagent: 100 g of molybdate free sodium tungstate are treated gradually with a solution of 30 ml. of "syrupy"

phosphoric acid (89 per cent.) in 150 ml of water. The mixture is boiled gently under reflux for 1 hour, decolorized as above with a drop of bromine, cooled and diluted to 500 ml.

Stock uric acid standard (Fohn) ($\equiv 1$ mg. per ml.): 1 g. of uric acid is placed in a 1-litre flask. 0.6 g of lithium carbonate is dissolved in 150 ml of cold water. The carbonate solution, filtered if necessary and warmed to 60° C., is added to the flask containing the uric acid, which is warmed under the hot tap. The warm mixture is shaken for five minutes, cooled at once under the tap, and treated with 20 ml. of formalin (40 per cent. solution of formaldehyde) and enough water to fill half the flask. A few drops of methyl-orange are added, and then gradually with shaking, 25 ml. of *N*-sulphuric acid. The solution should turn pink when 2-3 ml. of acid remain to be added. The mixture is now diluted to 1 litre, mixed and stored in the dark in a stoppered bottle, when it will keep almost indefinitely.

Uric acid "blood" standard ($\equiv 0.004$ mg. per ml.): 2 ml. of the above "stock" standard solution are diluted with water and 1 ml of 40 per cent formalin to 500 ml. This solution should be made up fortnightly.

Method: 0.2 ml of capillary blood is pipetted into 3.2 ml. of isotonic sodium sulphate in a 15 ml. centrifuge tube. 0.6 ml. of Fohn's (1934) uric acid reagent is added. The tube is stoppered, its contents mixed gently by inversion and immediately centrifuged. 2 ml. of the supernatant liquid ($\equiv 0.1$ ml. of blood) are treated with 1 ml. of sodium cyanide-urea reagent. At the same time a mixture of 1 ml of the uric acid "blood" standard ($\equiv 0.004$ mg. uric acid), 0.7 ml. of distilled water, and 0.3 ml. of Folin's reagent is treated also with 1 ml. of sodium cyanide-urea reagent. The two tubes are placed in a boiling water bath for 5 minutes, cooled, and the solutions compared colorimetrically.

Calculation:

$$\text{Uric Acid (mg per 100 ml.)} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 4.$$

5. CREATININE

Principle: Creatinine gives a red colour with alkaline picrate solutions (Jaffé's reaction). A similar colour is given with blood and plasma filtrates, but it is not certain that this colour is due to creatinine. Nevertheless, the substance estimated as blood "creatinine" is of clinical importance. The normal value for blood "creatinine" is 1-2 mg. per 100 ml. In renal failure, raised values may be found.

Reagents required:

Isotonic sodium sulphate, 10 per cent. zinc sulphate and 0.5 N-sodium hydroxide, as used for blood urea.

Creatinine stock standard (1 mg. per ml.): 1.602 g. pure creatinine zinc chloride are dissolved in N/10 HCl and volume made to 1 litre with N/10 HCl.

Creatinine "blood" standard (0.005 mg. per ml.): .5 ml. of stock standard and 10 ml. N/10 HCl are made up to 1 litre with water.

Alkaline picrate. Five parts (by volume) of a saturated aqueous solution of pure picric acid, containing about 15 g. picric acid per litre are mixed with 1 part (by volume) of 10 per cent. sodium hydroxide.

(*Note.*—The picric acid may be purified by recrystallization from glacial acetic acid. The colour given by a mixture of 10 ml. of the saturated aqueous solution and 5 ml. of 10 per cent. sodium hydroxide must not be more than twice as deep as that of the saturated picric acid solution itself.)

Method. 0.2 ml. blood is added to 1.4 ml. of isotonic sodium sulphate solution. 0.2 ml. of zinc sulphate and 0.2 ml. of 0.5 N-sodium hydroxide are added and the tube is stoppered and shaken. The mixture is centrifuged and 1 ml. of supernatant is used as "test" solution.

For normal blood, the standard is made by diluting 1 ml. of the creatinine "blood" standard to 5 ml. The test solution and 1 ml. of this standard are then treated at the same time with 0.5 ml. of freshly made alkaline picrate solution. After not more than 15 minutes, the solutions are compared in the colorimeter, using a blue-green filter, such as Ilford's spectrum blue-green (see p. 208).

Calculation:

$$\text{Blood creatinine (mg. per 100 ml.)} = \frac{\text{Reading of standard}}{\text{Reading of test}}$$

Where a raised blood "creatinine" is found, stronger standards may be used by diluting larger volumes of the blood standard to 5 ml. In general the calculation then becomes:

$$\text{Blood "creatinine" (mg. per 100 ml.)} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times C$$

where C is no. of ml. of blood standard used in 5 ml. of standard solution.

6. CHOLESTEROL

Principle: Blood is extracted with an alcohol-ether mixture, which at the same time precipitates the proteins. The extract is

8. INORGANIC PHOSPHATE

Principle: The inorganic phosphate of a deproteinized filtrate of the blood or plasma is coupled with molybdate, and the yellow phospho-molybdate is reduced to give a blue substance. The amount of blue colour produced in the solution is directly proportional to the amount of phosphate present.

Reagents required:

20 per cent trichloroacetic acid solution.

Ammonium molybdate: 5 g. of ammonium molybdate are added to a mixture of 75 ml distilled water and 15 ml. of concentrated sulphuric acid in a 100 ml volumetric flask. The mixture is shaken until dissolution is complete, and cooled to room temperature. The solution is then made up to 100 ml and mixed.

0.2 per cent aminonaphtholsulphonic acid: 0.2 g of the 1:2:4 aminonaphtholsulphonic acid, 12 g sodium metabisulphite, and 2.4 g. crystalline sodium sulphite are dissolved by shaking with enough water to make 100 ml. If the solution does not filter clear it should be left overnight and filtered again. A fresh solution should be prepared every two weeks. Tablets containing the correct amounts of 1:2:4 aminonaphtholsulphonic acid, sodium sulphite and sodium metabisulphite for 10 ml of solution are available from Messrs. Gallenkamp.

Standard phosphate: A stock solution is made by dissolving 2.194 g of pure potassium dihydrogen phosphate (KH_2PO_4) in 500 ml. in water. This solution contains 1 mg P per ml. A dilute standard solution is made by diluting 2 ml of the stock solution to 1 litre with water. 1 ml. of this solution contains 0.002 mg. P. Both solutions should be kept saturated with chloroform to prevent any bacterial growth, which might cause a loss of inorganic phosphate.

Method: 0.2 ml. of whole blood, serum or plasma, is pipetted into 3.2 ml. of water or isotonic sodium sulphate and treated with 0.6 ml of 20 per cent. trichloroacetic acid. The mixture is shaken well, and after 5 minutes filtered through a small paper. 2 ml of the clear filtrate (\approx 0.1 ml. of blood or plasma) are treated at the same time as 2 ml. of the dilute standard phosphate solution (\approx 0.004 mg. P) with 0.3 ml. of the ammonium molybdate solution followed by 0.02 ml of the reducing agent (aminonaphtholsulphonic acid). The contents of the tubes are gently shaken between each addition, and the colours are read after 10 minutes in a colorimeter.

Calculation:

$$\text{Phosphate (mg. per 100 ml.)} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 4.$$

9. SODIUM

Principle: This method is adapted from the procedure of Noyons (1939). After precipitation of the serum proteins by trichloroacetic acid the sodium in the filtrate is precipitated as sodium zinc uranyl acetate. After washing, this precipitate is treated with potassium ferrocyanide and the resulting plum red colour (uranyl ferrocyanide) is compared with that produced by a standard sodium chloride solution which has been treated in a similar fashion.

Reagents required:

Standard NaCl (containing 0.75 mg Na per ml) 191 mg of analytical dry sodium chloride dissolved in 100 ml in water in a volumetric flask.

Trichloroacetic acid: 7 g. per 100 ml in water

Zinc uranyl acetate reagent :—

20 g. of uranyl acetate, $\text{UO}_2(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, 60 g of zinc acetate, $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, and 60 ml. of glacial acetic acid are added to 320 ml. of distilled water and warmed gently until dissolved. After standing 24 hours the solution is filtered into a dark bottle and stored in the ice-box. The solution must be filtered immediately before use.

Saturated alcoholic sodium zinc uranyl acetate: 40 ml of zinc uranyl acetate reagent are mixed with 50 ml. of 50 per cent. alcohol saturated with sodium chloride; 100 ml. of absolute alcohol are added, and, after standing in the ice-box overnight, the supernatant solution is decanted. The precipitate is washed several times with alcohol, drained, dried and then shaken with 500 ml of absolute alcohol. This is stored in the ice-box and filtered immediately before use.

Potassium ferrocyanide: 20 g. dissolved in water and made up to 100 ml

Dilute acetic acid: 0.5 ml glacial acetic acid made up to 100 ml. with water

Method. To 0.5 ml. of serum are added 1.5 ml. of 7 per cent. trichloroacetic acid. The mixture is shaken well and filtered after 5 minutes; 0.2 ml. of the filtrate (\equiv 0.05 ml. serum) is transferred to a centrifuge tube containing 1 ml. of absolute alcohol and 0.4 ml. of zinc uranyl acetate reagent. The contents are mixed and kept in the ice-box overnight¹; they are then centrifuged for 15 minutes. The supernatant solution is then decanted, the tube allowed to drain on a filter paper for 10 minutes and the lip dried; 5 ml. of absolute alcohol saturated with sodium zinc uranyl acetate are added; the contents are mixed, by rotating the tube, centrifuged for 15

¹ It is not possible to obtain complete precipitation of the sodium zinc uranyl acetate in a short time. A 2-hour precipitation may be used, however, although the results will be less accurate.

minutes and drained as before. The precipitate is then dissolved by adding 10 ml. of dilute acetic acid; 0.25 ml. of potassium ferrocyanide solution is added and after mixing the tube is allowed to stand in the dark for 5 minutes. The coloured solution is compared with that produced from a standard sodium chloride solution, 0.2 ml. (\equiv 0.15 mg. Na) of which has been treated simultaneously in the same way as the deproteinized serum. (Colours must be read within 15 minutes or a clouding may occur.)

Calculation :

$$\text{Serum Sodium (mg. per 100 ml.)} = \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 300$$

$$\text{Serum Sodium (m. Eq./lit.)} = \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 130.5$$

Reference

NOYONS, E. C. *Pharm. Weekbl.* 76. (1939) 307.

10. POTASSIUM

Principle. The potassium is precipitated as cobalti-nitrite. The precipitate is washed, dissolved in hot water, and an excess of choline hydrochloride and sodium ferrocyanide is added. An emerald green colour develops immediately, the depth of which is proportional to the amount of cobaltous salt present, and hence to the amount of potassium. In view of the high potassium content of the cells, even traces of haemolysis must be avoided, and serum must be separated from the clot as soon as possible, certainly within 3 hours of taking the blood.

Reagents required:

Sodium cobalti-nitrite reagent : *Solution A :* 25 g. of cobaltous nitrate crystals are dissolved in 50 ml of water, and to this solution are added 12.5 ml. of glacial acetic acid. *Solution B :* 120 g. of sodium nitrite (potassium free) are dissolved in 180 ml. of water, giving a total volume of about 220 ml. To the whole of A are added 210 ml. of B. Nitric oxide is evolved. Air is drawn through the solution till all the gas has passed off. The reagent thus prepared is best kept in the ice-chest and should be filtered each time before use; it keeps for a month.

Standard potassium solution : 0.228 g of potassium sulphate (K_2SO_4) is dissolved in 500 ml. of water, giving a solution equivalent to 0.2 mg. K per ml.

Standard cobalt solution (\equiv 0.1 mg potassium per ml.) : 0.506 g. of cobalt ammonium sulphate is dissolved in a litre of water. Any

cobalt salt may be used but this is the easiest to weigh. The solution should be standardized against the standard potassium before use, but when once made is stable.

Choline chloride (1 g. per 100 ml.).

Sodium ferrocyanide (2 g. per 100 ml.).

Method: 0.5 ml. of serum (or of standard potassium solution) is placed in a conical centrifuge tube graduated at 4 ml. and 1 ml. of filtered sodium cobalti-nitrite reagent is added slowly with constant shaking. After 45 minutes 1 ml. of water is added, and the contents are mixed and centrifuged at moderate speed for 15 minutes. The tube is then inverted and drained briefly on filter paper; 2 ml. of water is added down the sides of the tube without disturbing the precipitate. The tube is again centrifuged for 5 minutes, inverted and thoroughly drained. The precipitate is washed with 5 ml. of 70 per cent. alcohol, and centrifuged and drained. The alcohol is blown into the tube so as to agitate the precipitate. 1 ml. of water is added and the tube placed in a boiling water bath until dissolution is complete.

In another graduated centrifuge tube is placed 1 ml. of the standard cobalt solution (0.1 mg. potassium); 0.5 ml. of choline chloride solution and 0.5 ml. of sodium ferrocyanide solution are added in that order to each tube and the volume made to 4 ml. The colours can be immediately compared in a colorimeter and are stable for several hours. (Orange or red filter.)

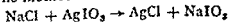
Calculation:

$$\text{Serum Potassium (mg. per 100 ml.)} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 20$$

$$\text{Serum Potassium (m Eq./lt.)} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 5.1$$

11. CHLORIDE

Principle: The method is based on the reaction



Silver iodate in ammoniacal solution is added to the deproteinized filtrate of blood or plasma. The excess of silver iodate, together with the silver chloride formed, is precipitated by the addition of acid, leaving in solution an amount of soluble iodate equivalent to the amount of chloride originally present. After the addition of potassium iodide, the amount of iodine set free from this soluble iodate is determined by titration with thiosulphate.

Reagents required:

Silver iodate reagent: Silver iodate is prepared by mixing equimolecular solutions of silver nitrate and potassium iodate. The precipitate is filtered, washed with distilled water, dried in vacuo, and preserved in the dark. 2 g. of the dried solid are dissolved in 100 ml. *N*-ammonia. Both silver iodate and its ammoniacal solution appear to decompose slightly when kept, with liberation of soluble iodate. Immediately before a series of determinations, therefore, 5 ml. of the stock (2 per cent.) ammoniacal silver iodate are acidified with 2 *N*-sulphuric acid (5 ml.) and centrifuged. The supernatant fluid is discarded and the iodate redissolved in 5 ml. of fresh *N*-ammonia.

10 per cent. zinc sulphate.

0.5 *N*-sodium hydroxide.

0.005 *N*-sodium thiosulphate.

Method: 0.2 ml. of serum or plasma is added to 1 ml. of water, 0.4 ml. of zinc sulphate solution and 0.4 ml. of 0.5 *N*-sodium hydroxide are added and thoroughly mixed. The mixture is then centrifuged. 1 ml. of the supernatant fluid (\equiv 0.1 ml. of plasma) is treated with silver iodate reagent (0.5 ml.) and, after mixing, with 2 *N*-sulphuric acid (0.5 ml.). The mixture is shaken and filtered through a small fine paper. 1 ml. of filtrate (\equiv 0.05 ml. of plasma), with the addition of 1 ml. of 1 per cent. potassium iodide, is titrated with 0.005 *N*-sodium thiosulphate, with starch as indicator.

Calculation:

$$\text{Chloride (mg. NaCl per 100 ml.)} = 97.5 \times \text{titre}$$

$$\text{Chloride (m.Eq./lit.)} = 16.7 \times \text{titre}$$

12. SERUM OR PLASMA PROTEINS

Principle: Serum or plasma diluted with isotonic saline is used for the estimation of total protein. A further sample is treated with sodium sulphite solution which precipitates the globulins and the filtrate is used for the estimation of albumin. For total protein and albumin, the protein is precipitated with zinc sulphate and sodium hydroxide, and the precipitate is digested with sulphuric acid containing selenium dioxide. Fibrin clot is similarly digested for the estimation of fibrinogen. The nitrogen content of the digests is estimated colorimetrically with Nessler's reagent. Nitrogen figures multiplied by 6.25 give the protein content, usually expressed as g./100 ml. of plasma or serum.

Reagents required:

50 per cent. sulphuric acid, containing 1.0 per cent. of selenium dioxide.

Calcium chloride: (For fibrinogen) 2.5 g per 100 ml. in water.

Sodium sulphite solution. 42 g of $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ dissolved in water and volume made to 100 ml.

Nessler's reagent, ammonium chloride standard, as used for non-protein nitrogen

10 per cent. zinc sulphate and 0.5 N-sodium hydroxide as protein precipitant.

Method—A. Total Protein: 0.2 ml. of plasma or serum is diluted to 10 ml. with isotonic sodium chloride (0.9 g/100 ml.). 0.2 ml. of this solution (= 0.004 ml. of plasma or serum) is added to 4 ml. water in a Pyrex centrifuge tube, 0.1 ml. zinc sulphate and 0.1 ml. 0.5 N-sodium hydroxide are added, the contents mixed and the tube centrifuged.

The supernatant is carefully decanted, and the inverted tube then drained on a filter paper. 0.2 ml. of 50 per cent. sulphuric acid containing selenium dioxide is then added, and a small piece of porous pot. The mixture is then boiled gently until blackening occurs and white acid fumes appear, and boiling is then continued for 15 minutes. To the cold, colourless solution are added 5 ml. of water and 3 ml. of Nessler's reagent. The colour is then compared with a standard prepared from 5 ml. of the standard ammonium chloride solution (0.01 mg. nitrogen per ml.), 2 ml. of water and 1 ml. of Nessler's reagent.

Calculation:

$$\text{Total Protein (A) (g./100 ml.)} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 1.25 \times 6.25$$

B. Albumin: A drop of octyl alcohol is placed in a 5 ml. volumetric flask. 0.2 ml. plasma or serum is pipetted into the flask and volume is made to 5 ml. with sodium sulphite solution. The flask is stoppered, well shaken and allowed to stand at room temperature for 10 minutes. The solution is then filtered through a fine filter paper. 0.2 ml. of filtrate (= 0.008 ml. of plasma or serum) is added to 4 ml. of water in a conical Pyrex centrifuge tube, and the solution mixed with 0.1 ml. zinc sulphate and 0.1 ml. 0.5 N-sodium hydroxide. The tube is centrifuged, the supernatant

containing selenium dioxide. After cooling, the colourless solution is treated with 5 ml. of water and 3 ml. of Nessler's

reagent and compared with the same standard as that used for total protein.

Calculation :

$$\text{Albumin (B) (g./100 ml.)} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 0.625 \times 6.25$$

C. Fibrinogen. To another 5 ml. of diluted plasma (= 0.1 ml. plasma) in a narrow tube is added 0.1 ml. of calcium chloride solution. The mixture is kept at 37° C. until clotting occurs. The fibrin is carefully collected on a thin glass rod, pressed to remove liquid and dropped into a test-tube for digestion. This and the colorimetric estimation is then carried out exactly as for total protein.

Calculation :

$$\text{Fibrinogen (C) (g./100 ml.)} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 0.05 \times 6.25$$

D. Globulin is calculated as follows :

For serum.

$$\text{Globulin} = \text{Total protein (A)} - \text{Albumin (B)}$$

For plasma.

$$\text{Globulin} = \text{Total protein (A)} - (\text{Albumin (B)} + \text{Fibrinogen (C)})$$

Copper Sulphate Method for Determining Plasma or Serum Protein

This method has been devised by Van Slyke and his colleagues and is of particular value in determining plasma or serum proteins rapidly in shock or wounds by measuring the specific gravity of plasma or serum in a series of copper sulphate solutions. By use of a more extensive range of solutions, approximate haemoglobin estimations can also be carried out. In pathological sera, with lipaemia or abnormal albumin/globulin ratios, the results are only approximate.

Principle : Drops of blood, plasma or serum are let fall into solutions of copper sulphate of known sp. gr. Each drop becomes encased in a sack of copper proteinate and remains as a discrete drop without change of gravity for 10 or 15 seconds, during which time its rise or fall shows its gravity relative to the solution. Temperature correction is unnecessary as the coefficients of expansion of CuSO_4 solutions approximate those of blood. The CuSO_4 solution cleans itself automatically after each test as the drop settles to the bottom.

Reagents: *Stock copper sulphate, sp. gr. 1.100:* 159.63 g. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (small crystals) are dissolved in water, and volume made up to 1 litre.

Standard copper sulphate solutions: A series of copper sulphate solutions of known specific gravity ranging from 1.033 to 1.018 is made by taking $n - 1$ ml. (where n = last two figures of specific gravity) stock copper sulphate and diluting to 100 ml. (e.g. for 1.033 standard, 32 ml. is taken). This operation is carried out at approximately the same temperature as that at which the stock solution was made; when dilute standards have been made, they may be used at any temperature. The solutions are kept in tightly screw-capped or stoppered bottles.

Method: A drop of plasma or serum is allowed to fall from a distance of about 1-2 cm. into solutions of appropriate specific gravity, and the movement for a few seconds after the drop has lost its initial momentum is observed; it can easily be determined that the specific gravity must lie between two adjacent figures.

The drops can be allowed to fall direct into the bottles, or the copper sulphate solutions can be poured into small test tubes and discarded after use. If the drops are put directly into the bottles, the 100 ml. of solution should be discarded after not more than 100 tests.

Calculation :

Protein concentration (g./100 ml.) = $360 \times (G - 1.0070)$

where G = sp. gr. of plasma or serum. A subtraction of 0.0004 from observed sp. gr. must be made for each mg. of oxalate per ml. of blood.

Reference

PHILLIPS, R. A., VAN SLYKE, D. D., DOLE, V. P., EMERSON, K., HAMILTON, P. B. & ARCHIBALD, R. M. U.S. Navy Research Unit, Rockefeller Institute (1945).

Another method of estimating plasma proteins by specific gravity is given in Chapter XIX.

13. SERUM PHOSPHATASES

Principle: The hydrolytic power of serum on sodium phenylphosphate buffered to pH of 4.9 and 10.0 respectively liberates phenol, and the amount of phenol set free is estimated by means of phenol reagent.

Reagents required:

Substrate. *M/100 disodium phenol phosphate*: 2.18 g. are dissolved in 1 litre of water, boiled to destroy moulds and organisms, and, after cooling, a few drops of chloroform are added as preservative. Keep in refrigerator.

Buffer (a) for "alkaline" phosphatase: 6.36 g. Na_2CO_3 (anhydrous) and 3.36 g. NaHCO_3 are dissolved in distilled water and made up to 1 litre.

(b) for "acid" phosphatase: 42.0 g. crystalline citric acid are dissolved in water: 376 ml. of *N*-NaOH are added, and volume made up to 1 litre. Keep in refrigerator.

Sodium carbonate: 20 g. Na_2CO_3 (anhydrous) are dissolved in warm water, and made up to 100 ml. Keep in warm place to prevent salt crystallizing out.

Phenol reagent (Folin and Ciocalteu): Dissolve 100 g. of sodium tungstate, $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, and 25 g. of sodium molybdate, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ in 700 ml. of water in a 1500-ml. Florence flask. Add 50 ml. of syrupy (85 per cent.) phosphoric acid and 100 ml. of concentrated hydrochloric acid. Connect the flask with a reflux condenser by means of a cork or rubber stopper wrapped in tin foil. Boil the solution gently for 10 hours. At the end of this time add 150 g. of lithium sulphate, 50 ml. of water and a few drops of liquid bromine. Boil the mixture without the condenser for about 15 minutes to remove the excess bromine. Cool, dilute to 1 litre and filter. The finished reagent should have no greenish tint. It should be kept well protected from dust, because organic materials will gradually produce slight reductions.

Dilute phenol reagent: The stock reagent is diluted 1 in 3 with distilled water.

Stock standard phenol: 1 g. pure crystalline phenol is dissolved in *N/10* hydrochloric acid and made up to 1 litre with *N/10* hydrochloric acid.

Standard phenol-plus-reagent (1 mg. phenol per 100 ml.): 5 ml. stock standard phenol are placed in 500 ml. graduated flask: 100 ml. dilute phenol reagent are added and solution made up to mark. This solution will keep 6 months in ice chest.

Method: Except for time of incubation and buffer used, the method for acid and alkaline phosphatase is identical.

Test: Into a conical centrifuge tube pipette 2 ml. of substrate and 2 ml. of appropriate buffer. Place the tube in 37° C. water bath for at least 3 minutes. Without removing tube from water bath add 0.2 ml. of cell free serum or plasma and leave tube in water bath for exactly the proper time (15 minutes for "alkaline," 3 hours for "acid," phosphatase). At the end of this time add 1.8 ml. of dilute phenol reagent and centrifuge.

Control. Exactly as-test, except that dilute phenol reagent is added *immediately* after serum or plasma, and mixture centrifuged.

Pipette 4 ml. of supernatant from test and control into separate test-tubes, and 4 ml. of standard phenol-plus-reagent into another tube. Add 1 ml. of 20 per cent. sodium carbonate to each tube and place tubes in 37° C. bath for 10 minutes to develop the colour. The colours are then read in the colorimeter; it is convenient to set the unknown at 30 and read the standard against it.

If the above method gives too high results, the incubation should be carried out for a shorter time, or serum should be diluted with saline, and the appropriate dilute serum control should be put up. The appropriate correction must be made in the calculation.

Calculation: The units in the two methods are different. For the "alkaline" phosphatase, the unit is the number of mg. phenol set free by 100 ml. serum in $\frac{1}{2}$ hour under the conditions of test: for the "acid" phosphatase the units is number of mg. phenol set free in 1 hour.

If the *unknowns* are set at 30 mm. the reading in mm. of standard against test (S_1) minus reading of standard against control (S_2) gives the number of mg. of phenol liberated per 100 ml. of serum.

With the standard times given above $S_1 - S_2$ is the actual unit value for "alkaline," but $\frac{S_1 - S_2}{3}$ is the value for "acid" phosphatase.

Normal Values:

Alkaline phosphatase 3-10 (occasionally up to 13) units per 100 ml.

Acid phosphatase 0.5-2.0 (occasionally up to 4.0) units per 100 ml.

Reference

GUTMAN, E. B & GUTMAN, A. B. *J Biol Chem.* 136 (1940) 201.

E. J. KING.

E. N. ALLOTT.



SECTION III
HAEMATOLOGY
AND
CYTOLOGY

CHAPTER XXII

HAEMATOLOGICAL NOMENCLATURE

THE terminology adopted by pathologists in classifying the cells found in the blood-forming organs and in the blood stream in pathological conditions is still rather confused and varies a great deal according to the school of thought to which the worker is accustomed. Such confusion is most noticeable in those medical communities in which more than one competent haematologist is in the field, and less where one stands prominently in the foreground as Naegeli in Switzerland or Ferrata in Italy.

This confusion originated when haematology was in its infancy and different "schools" were formed around workers such as Maximow, Pappenheim and Naegeli. Then various types of blood cells were observed almost for the first time, their characteristics noted and names given to them. Only too easily cells whose appearance was similar came to be called by different names (*lymphoidocyte, myeloblast, haemocytoblast, etc.*).

The method of staining the material under study has had much to do with the disparity of views. Most of the early work was done on histological sections while in more recent years the introduction of Romanowsky stains, supravital techniques and sternal puncture showed the same cells, so to speak, from a different angle and in different surroundings.

All workers in haematology have long since realized that not every cell that comes in the field of the microscope can be classified with certainty, and until more is known about blood formation in health and disease such cells will have to remain without definite names. On the other hand a study of the literature will show that many cells described under different names are actually of the same type.

Most workers nowadays, to whatever "school" they may belong, agree that the blood cells derive from the mesenchyme, and in particular from the reticulo-endothelium, and therefore admit the existence of a single type of cell which by division and maturation gives rise to all blood cells. It is generally believed that the development of the various blood cells takes place in different organs; erythrocytes, granulocytes and platelets in the bone-marrow, lymphocytes in the lymph glands, and monocytes mainly in the spleen: if one primitive stem cell is to be considered as the pre-

cursor of all blood cells then the environment in which this cell finds itself must decide the type of progeny.

Whether this stem cell once it has taken its fixed place in the body structure can give rise to all blood cells or only to some of them is a matter of opinion. Haematologists who claim to be able to recognize a myeloblast from a lymphoblast or monoblast will tend to adhere to the polyphyletic theory and admit the existence of several primitive stem cells, while those who follow the monophyletic theory will classify all primitive stem cells, always very similar in appearance, into one group only.

The Stem Cells

The term "myeloblast" has now come to have two different meanings. In the one case it is considered as the precursor of all blood cells (Naegeli, Downey), in the other as only the progenitor of the granulocytic series (Ferrata, Sabin and coll.). It is unfortunate on the one hand that a term etymologically meaning "a primitive marrow cell" should be applied only to one type of bone-marrow cells, but on the other hand the term myeloblast does bring to mind the idea of its successor, the myelocyte. Furthermore all descriptions of the myeloblast as generally understood stress the similarity between it and the lymphoblast or monoblast and state that recognition can be made only "by the company it keeps." This although probably accurate in the majority of cases may not be so in a few instances. It appears to us more reasonable to regard as *haemocytoblasts* all non-granular primitive stem cells and to reserve the term *myeloblast* for those cells which by Romanowsky, supravital or peroxidase stain show a definite development towards the myeloid series. Similarly we shall term lymphoblast or monoblast only those cells which can definitely be stated to possess lymphocytic or monocytic characteristics.

The Myeloid Series

The first indication that a cell belongs to the myeloid series is the appearance in the cytoplasm of granules which with the more commonly used Romanowsky stains appear of a deep red almost purple colour (azurophilic granules); they take up neutral red in supravital staining and are peroxidase positive. Ferrata and others consider these granules as non-specific, *i.e.* not yet indicating whether the cell will develop into a neutrophilic, eosinophilic or basophilic myelocyte. Sabin and her co-workers on the contrary

are of the opinion that these granules as seen in supravital preparations are indistinguishable from the granules of the mature neutrophilic polymorphonuclears. But other workers also using the supravital technique have noticed a difference in staining between the granules which appear in primitive cells and those of metamyelocytes and polymorphonuclears. It seems that the striking difference in appearance with the Romanowsky stains and with some special types of peroxidase stain warrants the differentiation of these granules into non-specific and specific (neutrophilic, eosinophilic and basophilic).

The Erythrocyte Precursors

Since Ehrlich gave the name of megaloblast to cells observed in foetal erythropoiesis and in some blood disorders, notably pernicious anaemia, this term has been used more and more to indicate the earliest recognizable immature precursor of the red cells; some workers consider that megaloblasts are a normal feature of the adult bone-marrow. In our opinion the megaloblast has such a particular and typical nuclear characteristic and is so constantly present in the foetal haemopoietic tissues and in the bone-marrow and peripheral circulation in pernicious anaemia, that it must be considered after Ehrlich's view as peculiar to foetal blood production and, in adult life, to disturbed haemopoietic activity.

The earliest recognizable precursor of the erythrocyte in normal bone-marrow we have termed proerythroblast, and have divided the immature red cells into two series, the normoblastic and the megaloblastic. In each of these with the maturation of the cell and the acquisition of haemoglobin, the nuclear characteristics remain practically unchanged except for a progressive coarsening and condensation of the chromatin up to pyknosis of the nucleus.

Other Blood Cells

Lymphocytes, monocytes, megakaryocytes, plasma cells and their precursors we have described as generally accepted.

Although there is little likelihood that in the near future one terminology will be universally adopted, it is hoped that the fairly simple nomenclature presented here may prove acceptable to the majority of workers in this field, and may serve as a basis for further discussion and an early settlement of the whole matter.

In describing and naming the immature blood cells we have followed the ideas outlined above. The staining properties of the

various cellular structures referred to are those observed in dry fixed films of material stained with the Romanowsky (Wright or Leishman) stains. The sizes mentioned are average ones; larger or smaller cells are frequently encountered especially in smears of pathological blood or bone-marrow.

CELL TYPES

Stem Cells

Haemohistioblast (20–25 μ): Large cell with irregularly outlined cytoplasm, faintly basophilic and often containing small azurophilic granules. The nucleus is round, with chromatin arranged as a loose net-work and a nucleolus more or less prominent.

Haemocytoblast (15–20 μ): Round cell with central or slightly eccentric nucleus; the cytoplasm is deeply basophilic and frequently shows a clearer zone shaped as a crescent at one side of the nucleus; no granules are visible in the cytoplasm. The nucleus is round with chromatin loosely arranged in a regular pattern; nucleoli are prominent and vary in number from one to five.

Myeloid Order

Myeloblast (14–18 μ): Round cell with nucleus frequently eccentric and slightly indented, the chromatin is loosely arranged and nucleoli are generally present. The cytoplasm is basophilic with a clearer zone in correspondence to the indentation of the nucleus and contains a few granules or short rods staining a deep purplish red.

Promyelocyte (14–18 μ): Round cell with central or slightly eccentric nucleus; the chromatin is coarser and arranged not so loosely as in the myeloblast; residua of nucleoli are sometimes seen. The cytoplasm is in parts basophilic and contains many non-specific granules staining deep red; in other parts it begins to take the appearance of the mature polymorphonuclear leucocyte with specific (neutrophilic, eosinophilic or basophilic) granules.

Myelocyte (12–16 μ): Round cell with round nucleus: the chromatin is coarse and begins to take the blotchy appearance of the nucleus of the polymorphonuclear leucocyte. The cytoplasm is very slightly basophilic and filled with specific granules. The non-specific deep red granules have disappeared or only the faintest trace of them is left.

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Metamyelocyte (10-15 μ): Round cell with horse-shoe or indented nucleus; the chromatin is coarse and arranged in lumps. The cytoplasm is filled with specific granules.

Band Form (10-12 μ): Similar to the preceeding but with the nucleus elongated in the form of a curved band of varying shape.

Polymorphonuclear Leucocyte (10-12 μ): Round cell with segmented nucleus. The chromatin is coarsely arranged in clumps. The cytoplasm is filled with specific granules (neutrophilic, eosinophilic or basophilic).

Lymphoid Order

Lymphoblast (15-20 μ): Large round cell with round nucleus. The chromatin is coarser and less homogeneous than that of the haemocytoblast and is concentrated at the edge of the nucleus giving the impression of a definite nuclear membrane. The nucleoli are prominent but rarely more than three are present. The cytoplasm is distinctly basophilic, contains no granules, and frequently shows a clear perinuclear zone.

Prolymphocyte (12-15 μ): Smaller than the lymphoblast still shows characteristics of immaturity of the nucleus with the chromatin not so densely arranged as in the lymphocyte and residua of nucleoli. The cytoplasm is basophilic but hyaline and transparent, frequently with perinuclear pallor and may contain azurophilic granules not peroxidase positive.

Lymphocyte (7-12 μ): Round cell with scanty cytoplasm basophilic and transparent, at times containing azurophilic granules. The nucleus is round, sometimes indented, with perinuclear membrane and the chromatin arranged in coarse masses.

Monocytic Order

Monoblast (18-24 μ): Large round or oval cell, occasionally with irregular outline. The cytoplasm is basophilic, more abundant than in the myeloblast or lymphoblast and does not contain granules. The nucleus is round, finely reticulated and showing numerous folds and convolutions: one or two nucleoli are present.

Promonocyte (16-20 μ): Transitional cell between the typical monocyte and the monoblast. The cytoplasm has a muddy appearance and contains few diffuse azurophilic granules faintly peroxidase positive. The nucleus is folded and wrinkled and appears coarser than that of the monoblast and contains no nucleoli.

Monocyte (14–18 μ) : Large cell with irregular outline. "Ground glass" lightly basophilic cytoplasm with numerous azurophilic granules and vacuoles. The nucleus is lobulated and horse-shoe shaped. The chromatin net-work is coarse but of regular pattern.

Erythroid Order

Proerythroblast (15–20 μ) : Large round cell with deeply basophilic cytoplasm devoid of granules, and a large round nucleus composed of finely reticulated chromatin with a tendency to form minute triangular masses, arranged roughly radially. Indistinct nucleoli are present.

Basophilic Normoblast (10–14 μ) : Round cell smaller than the proerythroblast with basophilic cytoplasm. Nucleus deeply stained, with chromatin coarsened and condensed by the coalescence of the net-work pattern, but still maintaining the radial arrangement.

Polychromatic Normoblast (8–12 μ) : The nucleus is composed of condensed masses of chromatin disposed in a cart-wheel arrangement. The cytoplasm is polychromatic due to the presence of haemoglobin.

Orthochromatic Normoblast (7–10 μ) The nuclear chromatin is condensed in a dark pyknotic mass of homogeneous appearance and the cytoplasm is fully haemoglobinized and acidophilic.

Basophilic Megaloblast (18–25 μ) : Large round cell with deeply basophilic cytoplasm sometimes with a paler perinuclear zone. The nucleus is large and has a chromatin net-work of very finely stippled minute knots and curved rods uniformly distributed throughout and separated by an even greater amount of slightly acidophilic parachromatin. Occasionally nucleoli are present and to such cells, evidently more primitive, the term Promegaloblast may be applied.

Polychromatic Megaloblast (12–16 μ) : Large cell with abundant polychromatic cytoplasm and nucleus showing the typical reticular pattern of the basophilic megaloblast though a little coarser.

Orthochromatic Megaloblast (10–15 μ) : Smaller cell than the preceding, with cytoplasm fully haemoglobinized and acidophilic and a small nucleus frequently eccentric but still showing the reticular arrangement of the megaloblastic pattern.

Other Cells

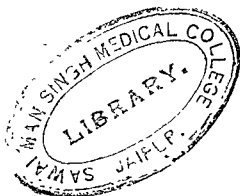
Plasma Cell (12-16 μ): Round cell with eccentric nucleus. Cytoplasm intensely basophilic with a paler zone surrounding the nucleus, at times containing azurophilic granules or vacuoles. The nucleus is round or oval, with the chromatin coarsely clumped in a cart-wheel arrangement.

Megakaryocyte (25-40 μ): Large ameboid cell with pale basophilic cytoplasm often containing azurophilic granules. Nuclear structure complex, appears to consist of many nuclei variously arranged, generally devoid of nucleoli.

B. L. DELLA VIDA.
F. PICK.

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CHAPTER XXIII

THE MYELOGRAM AND ITS CLINICAL APPLICATIONS

WITHIN the past 25 years bone-marrow biopsy by making possible the study of the life history of the blood cells has provided an important contribution to the understanding of the mechanism underlying changes in the peripheral blood and has given valuable help in the diagnosis and prognosis of "blood" disorders.

The diagnosis of many "blood" disorders may be made by examination of the peripheral blood alone, but in some study of the bone-marrow is indispensable for accurate diagnosis and in others is of confirmatory value and prognostic significance.

DISTRIBUTION AND STRUCTURE OF THE BONE-MARROW

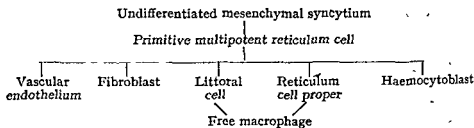
At birth the liver and spleen cease to be the sites of active haemopoiesis and the bone-marrow of the flat and long bones becomes the centre of blood formation. After the age of 7 years the pink-red marrow is slowly replaced by the fatty marrow; from the age of about 20 active haemopoietic marrow remains only in sternum, ribs, clavicles, scapulae, vertebrae, the cranial diploë, the flat bones of the pelvis and the upper ends of the femur and humerus. Under pathological stimulus red marrow may replace the inactive fatty marrow, and the same stimulus may provoke extra-medullary haemopoiesis in the whole of the reticulo-endothelial system.

The bone marrow consists of medullary tissue supported by a delicate interlacing framework of reticulin fibrils. The medullary tissue consists of undifferentiated mesenchymal syncytium, primitive multipotent reticulum cells, derived from the syncytium, and more highly differentiated cells. These last are: the reticulum cells proper or fixed histiocytes, through the body of which run the reticulin fibrils; the littoral cells lining the sinuses and intersinusoidal capillaries and potentially phagocytic; wandering cells or macrophages (polyblasts); fibrocytes; immature and mature blood cells. Between the meshes of the supporting framework of reticulin fibres lies a vast sinusoidal network of thin-walled vessels, the branches of the nourishing artery, lined by littoral and endothelial cells. The reticulin fibrils condense to form the walls of intersinusoidal capillaries, lined by littoral and primitive reticulum (endothelial) cells, which intercommunicate with the sinusoidal vascular system.

The primitive reticulum cell is multipotent, non-motile, does not produce reticulin fibrils and does not store dyes. Under various stimuli it can differentiate either into fixed histiocyte, littoral cell, macrophage, vascular endothelium, fibrocyte or primitive blood cell (haemocytoblast). Only the endothelial cells of the sinuses of the bone marrow, spleen, liver and lymph-node of the adult preserve haemopoietic potency.

The reticulum cell proper (tissue histiocyte, resting wandering cell) is a more differentiated cell, potentially phagocytic and dye storing. Under appropriate stimulus it may free itself, become motile and highly phagocytic (macrophage, polyblast) or may differentiate into a fibroblast. Littoral cells (littoral histiocytes) of the sinuses of the bone marrow, spleen, liver and lymph-nodes also possess this mesenchymal potency. Littoral cells lining the rest of the vascular system have little or no phagocytic properties.

The following scheme shows the derivation of cells from the undifferentiated mesenchyme.



Methods of Studying Bone Marrow

The sternal bone marrow is now usually chosen for examination in adults, the tibial in children. Two methods are available for obtaining specimens: (a) the trephine; (b) the aspiration method.

(a) This is a surgical operation; it provides material both for sections and smears. With a 1 cm. trephine a disc of bone with attached marrow is removed, usually from the shaft of the tibia; fragments of bone marrow are curetted and smears and imprints made on glass slides, the tissue is fixed, decalcified, sectioned and stained in the usual way. By this method the architecture and the relative proportion of the various cells are preserved and new growth, aplasia and fibrosis can readily be detected.

(b) Sternal puncture is for ease of performance and safety the method of choice. It is a simple, painless bedside procedure which can be repeated at frequent intervals with no subsequent scarring and minimal risk of infection.

Technique of Sternal Puncture: The sternal puncture instrument should be a small strong needle with stylet and movable guard to prevent too deep penetration. Many types are on the market; those of Salah or Witts are suitable. The patient lies supine; after cleansing the site of puncture with iodine and alcohol, the skin, subcutaneous tissue and periosteum are infiltrated with about 2 cc. of 1 per cent. novocaine solution; after 3-5 minutes the sterile puncture needle is introduced. When the point of the needle touches the bone, the guard is adjusted and fixed about half an inch (1 cm.) above the skin. Puncture may be made into either:

- (1) The body of the sternum at the level of the second or third intercostal space, slightly to one side of the mid-line, or
- (2) The manubrium sterni in the mid-line just above the manubrium-gladiolar juncture.

In the first case the needle is pushed firmly into the marrow cavity at a right angle with the plane of the sternum; in the second after penetration of the outer plate, the angle of the needle is altered so that it points towards the patient's head. When it remains firmly fixed without support the needle is in the marrow cavity. The stylet is then withdrawn and a 5 or 10 cc. dry syringe affixed. Moderate suction is applied and 0.25-0.5 cc. of the red marrow mixed with blood is withdrawn. The syringe is detached, the stylet reinserted and the needle removed. From the material in the syringe direct films of varying thickness are immediately made and the remainder discharged into an anti-coagulant, preferably Heller and Paul oxalate mixture (see p. 291).

Technique of Tibial Puncture: Tibial puncture is to be used for preference in infants and small children: the same instrument, sterile precautions and local anaesthesia are as essential as for sternal puncture.

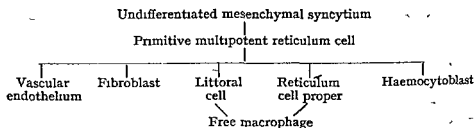
The marrow of the upper end of the shaft of the tibia is entered about 2 cm. below the anterior tibial tuberosity which in infants represents the line of the epiphysis, in the centre of the medial subcutaneous plate and at right angles to it. When the limb is semi-externally rotated the bone surface will lie in a horizontal plane. In fat infants the point of the needle may have to travel from the surface of the skin for a distance of 2-2.5 cm. before entering the marrow cavity.

When information as to the architecture of bone and marrow is required the trephine method should be used. Marrow puncture supplies the means of evaluating gross alterations in the distribution

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When information as to the architecture of bone and marrow is required the trephine method should be used. Marrow puncture supplies the means of evaluating gross alterations in the distribution

frequency of the cells and of studying their morphology. For clinical purposes this is usually what is mainly required.

Examination of Specimen: In order to avoid autolysis all material should be fixed within half an hour of withdrawal; for the same reason postmortem specimens should be taken within half an hour of death and fixed at once. Methyl alcohol is the fixative of choice.

The main value of the examination of the bone marrow attaches, as previously pointed out, to analysis of departures from normal, in particular as to morphology and distribution frequency of the formed elements other than the erythrocytes. Such an analysis is arrived at by constructing a chart of the distribution of the nucleated cells or Myelogram: for this purpose it is necessary to have a clear idea of the varieties of the cells to be charted and of their morphology. The types of cells to be included in the Myelogram are shown below. For description of these cells, see p. 237 *et seq.*

Several films should always be prepared and examined and there should be no significant variation in the differential count of each film. At least 500 cells should be counted.

The ratio of the cells of the myeloid to those of the erythroid order naturally varies with the relative activity of the two cell orders and is one of the most valuable pieces of information provided by the Myelogram. This ratio known as the myeloid-erythroid ratio (M.-E.R.) normally varies from 8 : 1 to 2 : 1 with an average of 3.5 : 1. A further means of estimating the relative activity of the two orders of haemopoietic cells is by the calculation of the ratio of immature granulocytes (myeloblasts to "band" forms) to nucleated red cells. This is known as the leuco-erythrogenic ratio (L.-E.R.). The average normal figure lies between 1.5 : 1 to 2.5 : 1.

The following Myelogram represents an average normal :

	Minimal range	maximal %	Average %	
Haemohistioblast (reticulum cell)	0.0-1.0		0.50	
Haemocytoblast	0.0-1.0		0.50	
Myeloblast	0.2-4.0		1.00	} 47.25 66.50
Promyelocyte	0.5-10.0		3.00	
Neutrophil myelocyte	2.0-20.0		13.00	
Eosinophil myelocyte	0.3-2.0		1.00	
Basophil myelocyte	0.0-0.5		0.25	
Metamyelocyte	5.0-18.0		14.00	
Band form	5.0-30.0		15.00	
Polymorphonuclear leucocyte	9.0-34.0		18.00	
Eosinophil	0.5-3.0		1.00	
Basophil	0.0-0.5		0.25	

	Minimal maximal range %	Average %
Lymphocyte	2.5-24.0	10.00
Monocyte	0.0- 5.0	2.00
Plasma cell	0.0- 2.0	0.50
Megakaryocyte	0.0- 2.0	0.25
Proerythroblast	0.0- 2.0	0.50
Normoblast basophilic	0.3- 3.0	2.00
Normoblast polychromatic	5.0-15.0	12.00
Normoblast orthochromatic	1.0- 7.0	5.00
Cells in mitosis	0.1- 0.4	0.25
Smudge cell (disintegrated cell)	0.3- 2.0	

19.50

Myeloid-erythroid ratio 8 : 1-2 : 1	M-E R. = $\frac{66.50}{19.50} = 3.4 : 1$
Leuco-erythrogenic ratio 1.5 : 1-2.5 : 1	L-E R. = $\frac{47.25}{19.50} = 2.4 : 1$

THE ANAEMIAS

Recent studies of normal and pathological erythropoiesis serve to correlate bone marrow changes with anaemias of different aetiological and clinical type. Following Israëls' classification (1941), four main types of pathological erythropoiesis characterized by four classes of bone marrow pictures can be recognized. Most of the anaemias and their respective myelograms will be found to fall into one or other of the four groups.

- (I) Failure of maturation of proerythroblasts with suppression of normoblastic development and its replacement by megaloblastic hyperplasia and production of large red cells (megalocytes).
- (II) Failure of maturation of normoblasts with hyperplastic but distorted development of inadequately haemoglobi-nized red cells (microcytes).
- (III) Hyperplastic normal maturation with increase of pro-erythroblasts and normoblasts.
- (IV) General hypo- or aplasia of erythropoietic tissue.

Class I: This type of bone marrow picture appears to be associated with deficiency in the production, utilization, mobilization or assimilation of the hypothetical anti-anaemic principle resident in the liver. The resulting anaemia is megalocytic and normo- or hyperchromic. Megaloblasts may be found in blood films.

Of the types responsive to liver the principal are: Pernicious anaemia, tropical nutritional macrocytic anaemia, the "pernicious anaemia" of pregnancy, the anaemias of sprue and idiopathic steatorrhoea, that associated with some diseases, operations upon and malformations of the intestinal tract and the anaemia of *Diphyllobothrium latum* infestation.

Myelogram: The striking feature is the presence of promegaloblasts and megaloblasts, normally altogether absent from the bone marrow, in numbers of up to 50 per cent. of the total nucleated cells. The M.-E.R. shows a pronounced predominance of the cells of the erythroid order.

In general anaemias associated with a megaloblastic marrow respond to liver therapy; an exception is the megaloblastic anaemia refractory to liver treatment, described by Israëls and Wilkinson (1936-1940) as "achrestic anaemia."

Class II: The anaemia in this class is micro- or normocytic and hypochromic. The main clinical types are idiopathic hypochromic (iron deficiency) anaemia, the hypochromic anaemia of pregnancy, chlorosis and the anaemias of infancy and childhood.

Myelogram: An increase in erythropoiesis which is of normoblastic type and roughly proportional to the degree of anaemia. The dominating cell is a small polychromatic normoblast with scanty irregular rim of grey cytoplasm.

Class III: The clinical picture associated with this type of bone marrow is shown by two groups of anaemias, A and B below:

A. (1) Anaemia due to loss of blood (haemorrhagic).

Myelogram: hyperplasia of erythroid and to a lesser degree of myeloid tissue; a striking increase in basophilic and polychromatic normoblasts, which are larger than usual with a cytoplasm suggesting early haemoglobinization and leading to the production of large red cells (macrocytes). In chronic cases bone marrow may become hypoplastic or aplastic.

(2) Haemolytic anaemias (idiopathic, congenital or secondary).

Myelogram: extreme hyperplasia of erythropoietic tissue with marked increase in proerythroblasts and basophilic normoblasts. Three-quarters of the marrow cells may be nucleated red cells and

a high proportion up to 80 per cent. are present as reticulocytes. Relative granulocytopenia is usual. Infective and toxic group of haemolytic anaemias usually produce a milder marrow reaction. Protracted cases may come to aplasia of bone marrow.

(3) Anaemias associated with cirrhosis of the liver, leukaemia and malignancy.

B. Anaemias of unknown aetiology, resistant to treatment (pseudo-aplastic) frequently macrocytic.

Some of the patients belonging to this group show temporary or permanent restoration of blood picture, either spontaneously or after repeated blood transfusions. Many of the cases described by Bomford and Rhoads (1941) under the name of "refractory anaemia" appear to have had a bone marrow and a clinical picture which fall under this heading.

Myelogram: Decrease of more mature granulocytes; in the erythroblastic series, an increase in normal basophilic and polychromatic normoblasts and often a number of abnormally large normoblasts, the cytoplasm of which shows early haemoglobinization. The maturation of these giant normoblasts determines the macrocytic character of the peripheral blood picture. These cases generally do not respond to liver therapy; the examination of the bone marrow is of diagnostic importance in differentiation from megaloblastic anaemia.¹

Class IV: Myelograms in this type of cases fall, according to Whitby and Britton (1942), into two categories.

These are:

- (1) True aplasia with failure of division of primitive blood cells.
- (2) Maturation defect with failure in production and delivery of mature cells.

In nearly all cases not only the erythropoietic but also the leucopoietic and thrombopoietic tissues are affected though in variable

¹ In recent years Di Guglielmo has collected several cases, some acute and some chronic, of anaplastic hyperfunction of the erythropoietic system, and has attempted to unite them in a group analogous to the leukaemias. The *erythraemic diseases*, which include Cooley's anaemia and Vaquez' polycythaemia, present on the whole a myelogram which falls under the heading Class III B; there is constant involvement of the reticulo-endothelial system which acquires both haemopoietic and haemolytic activities; in view of this these forms should properly be classified under the Reticuloses — (Ed)

DI GUGLIELMO, G., *Haematologica*, 17. (1936) 19

— & QUATTRIN, N., *Haematologica*, 24. (1943) 1.

BONE MARROW EXAMINATIONS

AVERAGE MYELOGRAMS OF

	Normal	Pernicious anaemia (relapse)
Reticulum cell . .	0.0-1.0	0.5-2.5
Haemocytoblast . .	0.0-1.0	0.2-3.0
Myeloblast . .	0.2-4.0	0.2-4.0
Promyelocyte . .	0.5-10.0	1.0-12.0
Neutr. myelocyte . .	2.0-20.0	12.0-20.0
Eosin. " . .	0.3-2.0	0.5-2.0
Basoph. " . .	0.0-0.5	—
Metamyelocyte . .		
Band forms	10.0-48.0	5.0-23.0
Polymorph . .	9.0-34.0	3.0-12.0
Eosinophil . .	0.5-3.0	1.0-2.0
Basophil. . .	0.0-0.5	—
Lymphocyte . .	2.5-24.0	6.0-13.0
Monocyte . .	0.0-5.0	0.0-2.0
Plasma cell . .	0.0-2.0	—
Megakaryocyte . .	0.0-2.0	—
Proerythroblast . .	0.0-2.0	0.5-3.0
Basoph. normoblast . .	0.3-3.0	10.0-20.0
Polychr. " . .	5.0-15.0	
Orthochr. " . .	1.0-7.0	
Mitosis . .	0.1-0.4	0.2-2.0
Promegakaryoblast . .		3.0-10.0
Basoph. Megaloblast . .		2.0-15.0
Polychr. " . .		4.0-25.0
Orthochr. " . .		3.0-10.0
Myeloid-erythroid ratio	8:1-2:1	2:1-0.5:1
Leuco-erythrog ratio	1.5-2.5:1	0.3-1.6:1

degree. Only a few cases of anaemia due exclusively to failure of the erythropoietic tissue have been recorded; some of the "refractory anaemias" of Bomford and Rhoads fall into this group.

Failure of the myeloid and thrombopoietic tissues will be mentioned in a later section (p. 253).

(1) *Aplastic anaemia (true aplasia)*. Myelogram shows a variable degree of hypoplasia of all the bone marrow tissues though a few atypical normoblasts may be present; more striking is the paucity of myelocytes, degenerative changes in polymorphs and finally the appearance of bizarre cells, very difficult to classify; absolute or

BY STERNAL PUNCTURE

DIFFERENT ANAEMIAS

Haemorrhagic anaemia	Iron deficiency anaemia	Haemolytic anaemia	Idiopathic aplastic anaemia
0.0-1.0	0.0-1.0	0.0-1.0	0.0-0.8
0.2-4.0	0.0-1.0	0.2-4.0	0.0-0.3
0.2-1.0	0.2-4.0	0.2-1.0	0.1-0.4
0.5-5.0	0.5-5.0	0.5-15.0	0.2-3.0
2.0-15.0	2.0-10.0	2.0-15.0	1.0-7.0
0.5-2.0	0.0-1.0	0.5-2.0	0.2-1.0
—	—	—	—
5.0-20.0	5.0-15.0	5.0-20.0	4.0-16.0
5.0-10.0	5.0-15.0	5.0-15.0	5.0-10.0
1.0-2.0	0.0-1.0	1.0-2.0	0.0-0.5
—	—	—	—
2.5-15.0	2.5-15.0	3.0-15.0	30.0-50.0
0.0-3.0	0.0-3.0	0.0-3.0	3.0-8.0
—	—	—	—
—	—	—	—
0.5-3.0	0.5-2.5	1.0-5.0	0.0-1.0
1.0-10.0	1.0-10.0	5.0-15.0	3.0-10.0
10.0-20.0	15.0-35.0	10.0-30.0	
10.0-15.0	1.0-10.0	10.0-15.0	
0.2-0.8	0.2-0.8	0.2-2.0	0.0-0.1
3:1-1:1	4:1-1:1	2:1-1:1	8:1-1:1
0.4-1.6:1	0.5-1.5:1	0.2-0.5:1	1.0-2.5:1

relative increase in lymphocytes and relative increase in monocytes and plasma cells is usually present. In the symptomatic aplastic anaemias, due to the action of various drugs and acute or chronic infections, the changes are similar though the myeloid tissue is attacked first. In suspected cases in which the peripheral blood may show only a mild degree of anaemia, the myelogram may give early information of hypoplasia of the myeloid tissue.

(2) *Aplastic anaemia (maturation type)*. Represented by the last stage of anaemias due to deficiency of maturation factors.

THE RETICULOSES

The Leukaemias

The white cells of the blood derive from the reticulum cells of the haemopoietic system; under the stress of abnormal stimuli any part of the reticulo-endothelial system may become the site of active proliferation of the white blood cells.

It follows that any such departure from normal of the reticulo-endothelial system will fall under the heading of the Reticuloses. In particular, following Robb-Smith (1938) most of the white blood cells dyscrasias are to be classified in the group of the Medullary Reticuloses.

The condition of leukaemia, leukaemic reticulosis or leukosis is usually readily identified from the examination of the peripheral blood. In certain cases (aleukaemic leukaemia) or in certain phases of the disease however the immature cells may remain locked up at their site of origin; in such cases the diagnosis of leukaemia from the examination of the peripheral blood may be impossible; it is readily made from examination of the marrow.

Myeloid Leukaemia

Myelogram: the myeloid cells are greatly increased with a corresponding rise of the M.-E.R. In chronic cases eosinophilic and basophilic myelocytes are very numerous; the proportion of myeloblasts and promyelocytes increases with the severity of the condition. Immature red cells are diminished. The more acute the condition the more do the myeloblasts and even more primitive "stem" cells come to dominate the picture.

Aleukaemic phases may occur in patients while under X-ray therapy. In such cases it is essential to know whether the aleukaemia is due to aplasia of the bone marrow as a result of excessive irradiation or whether it represents a spontaneously occurring phase in the disorder. In the former case further irradiation is contra-indicated; in the latter it may be carried on with impunity. Examination of the marrow offers the only means of arriving at a decision.

Chloroma may be regarded as a type of myeloid leukaemia in which for the most part the abnormal chloroma cells do not gain ingress to the blood stream but remain localized in the bone marrow. Examination of the bone marrow is of value in diagnosis. The

chloroma cells are large, with basophilic cytoplasm and bulky reticular nucleus.

Eosinophilic Leukaemia (Scott, 1939) may be regarded as a sub-type of myeloid leukaemia. The myelogram shows a very high proportion of eosinophilic promyelocytes and myelocytes.

Myelo-sclerosis : Chronic cases of myeloid leukaemia may pass into the condition of Myelo-sclerosis or this may arise *de novo*. The blood picture is that of a leucoerythroblastosis characterized by the presence of immature cells of the myeloid order together with many normoblasts. In these cases the unknown abnormal stimulus appears to affect the reticulum cell in such a way as to bring about abnormal proliferation both of the fibroblasts and of the primitive blood cells of the myeloid and erythroid series alike. There is resulting sclerosis of the bones; this may be patchy and only identifiable by X-rays. Examination of the bone-marrow shows replacement of the haemopoietic tissue by fibrous tissue within which occur areas showing abnormal proliferation of immature cells both of the myeloid and erythroid orders. In most cases the bone marrow cannot be obtained by sternal puncture; the trephine must then be used.

Lymphatic Leukaemia

The diagnosis is usually readily made from examination of the peripheral blood but as in the case of myeloid leukaemia aleukaemic forms may occur and can only be identified by examination of the bone marrow; this shows replacement of the myeloid and erythroid by lymphoid elements. In more chronic cases these consist of fully formed small lymphocytes; with increasing acuteness there is a rise in the proportion of lymphoblasts and an increase in the proportion of cells in mitosis.

Lymphosarcoma may be regarded as a type of reticulosis in which, as in chloroma, the abnormal cells fail to appear in the blood stream in large numbers. Kato and Brunshwig (1933) have reported cases of lymphosarcoma which after therapeutic irradiation developed the blood picture of lymphatic leukaemia. As a rule lymphosarcoma shows no specific blood picture or myelogram.

Monocytic Leukaemia

This always runs an acute or sub-acute course over a matter of a few weeks or at most a few months; chronic cases do not occur. The abnormal cells may not enter the peripheral blood in large numbers; the white cells may not rise above 20,000

per c.mm. at any stage of the disease and diagnosis may be impossible without examination of the marrow. The myelogram is characteristic, showing hyperplasia of the reticulum cells with great increase in monocytes and their precursors and suppression both of myelopoiesis and erythropoiesis. On account of the close resemblance between the precursors of the cells of the monocytic and lymphoid orders it may be necessary to have recourse to peroxidase staining; monocytes and most of the promonocytes show poor but definite peroxidase staining thus differentiating themselves from all the cells of the lymphoid order. It is probable that monocytic leukaemia bears the same relationship to reticulum cell sarcoma as does myeloid leukaemia to chloroma and lymphatic leukaemia to lymphosarcoma.

Plasma Cell Leukaemia

About fifteen cases have been described under this name. If it actually occurs it probably bears the same relationship to myelomatosis as does myeloid leukaemia to chloroma, lymphatic leukaemia to lymphosarcoma, and monocytic leukaemia to reticulum cell sarcoma.

Myelomatosis: When generalized it is readily diagnosed by sternal puncture; even in cases of solitary tumours in other parts of the skeleton the sternal marrow often shows foci of typical myeloma cells. These are large and irregular with basophilic cytoplasm containing sometimes vacuoles and azurophilic granules and showing generally a clear perinuclear zone. The nucleus is comparatively small and usually eccentric; the chromatin has either the "cart-wheel" appearance or is irregularly arranged in dense masses. Large clear nucleoli are present and mitotic figures are often seen. Bizarre forms resembling lymphocytes, myeloblasts or erythroblasts may be present in varying numbers. Controversy exists as to the nature of the myeloma cells. Piney (1943) described four types; true plasma cell, myeloblastic, lymphoblastic and erythroblastic. Rohr (1937) and Klima (1938) suggest that the myeloma cell derives from the reticulum cell and that the different cells appearing in the various types of myeloma represent stages in development.

Hodgkin's Disease

The blood picture and myelogram are not characteristic: typical "mirror image" giant cells (Sternberg-Reed) may be found but may also occur in other types of reticulososes.

The Lipidoses (Storage Reticuloses)

In all these conditions examination of the bone marrow may reveal the typical lipid containing reticulo-endothelial cells. Negative findings however do not exclude the diagnosis.

Gaucher's Disease: Bone marrow shows hyperplasia of erythroid and myeloid tissues and a slight increase of monocytes and eosinophiles. In the majority of cases the typical Gaucher cells are found either singly or in clusters of 3-8 cells; these are large, 15-80 μ in diameter, polygonal or elongated, with abundant acidophil or colourless cytoplasm, containing a fibrillar network, with one or more nuclei, usually dark, pyknotic and eccentric in location. The least differentiated cells have the nucleus characteristic of reticulum cells.

Niemann-Pick Disease: Bone marrow shows hyperplasia of myeloid tissue, and large masses of yellowish foam cells, 30-60 μ in diameter, with reticular nucleus and cytoplasm filled with fine foamy droplets.

Essential Xanthomatosis (Hand-Schüller-Christian Syndrome): the bone marrow may be extensively affected and sternal puncture may reveal cells similar to those found in Niemann-Pick Disease. On the whole, splenic puncture is probably more reliable than bone marrow aspiration in the diagnosis of storage reticulosis.

Miscellaneous

Agranulocytosis (granulophthisis, aleukia): can be divided into primary or idiopathic and secondary, due to action of drugs such as sulphonamides, severe infections, or excessive dosage of X-ray or radium. Both these conditions may occur as aplastic or maturation types (see p. 248). In primary cases the myelogram shows almost complete disappearance of immature white cells and diminution of polymorphs with excessive lobulation and degeneration of the survivors. In secondary cases the myelogram shows a normal or increased number of immature polymorphs with varying diminution of mature forms. In both cases erythropoiesis is either normal or only slightly suppressed. Sternal puncture is of value in differentiating this condition from aplastic anaemia and aleukaemic leukaemia, and in distinguishing the two types of agranulocytosis. The prognosis is much more favourable in the secondary than in the primary type.

Thrombocytopenia (idiopathic or symptomatic): may occur as aplastic or maturation type. In the latter case the myelogram

shows normal or increased number of atypical megakaryocytes and splenectomy often proves beneficial.

Metastatic Neoplasms : metastases from malignant tumours may occur in the bone marrow in the form of aggregations of atypical cells ; in only a few cases are the characteristics of the primary growth retained. Varadi (1937) reported a case of primary growth of the liver diagnosed by costal puncture. Most of the metastases are from prostate, breast or gastric carcinomata. Apart from the collections of atypical cells the bone marrow invariably shows a leukaemoid reaction : in some cases this reaction is so widespread that it shows itself in the blood in the form of leucoerythroblastic anaemia.

Infectious Mononucleosis : does not present a typical bone marrow picture although in many cases an increase in lymphocytes, plasma cells and abnormal monocytes is found. Marrow puncture may prove useful in excluding lymphatic leukaemia (see p. 301).

Tropical Diseases : Sternal puncture may give valuable information in certain tropical diseases.

Kala-azar : *Leishmania Donovanii* may in many cases be readily demonstrated within the monocytes of the marrow obtained by sternal puncture. This is easier, and safer than either splenic or hepatic puncture and appears to give equally good results.

Malaria : Recent reports (Aitkin, 1943 ; Rumball, 1943) indicate that examination of the marrow obtained by sternal puncture offers a better means of identifying the malarial parasite than examination of the blood either by thin or thick film.

Sternal puncture has also proved its value in the diagnosis both of filariasis and histoplasmosis

Bacterial Infections : Recent observations indicate that infecting organisms may be grown from the bone marrow even more readily than from the peripheral blood. Thus in 110 cases of typhoid fever Ling (1940) obtained the organisms from the sternal marrow in 44 cases in which he was unable to get a growth from the blood, in one case on the sixty-seventh day of the illness. McDonald (1941) likewise isolated *B. paratyphosum B.* from the marrow after it had disappeared both from peripheral blood and faeces. Falconer and Leonard (1941) in a case of infective endocarditis grew *Streptococcus viridans* from the bone marrow after four negative cultures of the peripheral blood.

F. PICK.

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CHAPTER XXIV

THE Rh FACTOR

THE discovery by Landsteiner and Wiener (1940) of a previously unrecognized antigen "Rh" in human erythrocytes was rapidly followed by demonstration of its importance in blood transfusion (Wiener *et al.*, 1940) and in the aetiology of haemolytic disease of the foetus (Levine *et al.*, 1941). Since then much additional information has accumulated. In this chapter an attempt is made to summarize the present position.

The presence of Rh factor in human erythrocytes was first demonstrated by testing different human bloods against an anti-rhesus serum, prepared by injecting the erythrocytes of rhesus monkeys into rabbits. This approach, in the search for new individual properties in human blood, was adopted because it was already known that the blood of some animals contains antigens related to the agglutinogens of human erythrocytes. It was found that while the red cells of the majority of human bloods were agglutinated by this anti-rhesus serum a small proportion were not. Evidently then some contained a "Rhesus" antigen (Rh Positive bloods) and some did not (Rh Negatives). It was then shown that the distribution of the Rh antigen amongst different human beings was quite independent of the distribution of the ABO groups and MN types.

Distribution in Different Populations

Amongst the American white population tested there were found to be about 85 per cent. of Rh positives and 15 per cent. of Rh negatives (Wiener *et al.*, 1940). A similar incidence has been found both in England and Germany. As with other blood group factors there are marked racial differences in distribution. For instance, American Indians, Chinese and Australian Aborigines are all, or almost all, Rh positive.

Relation to Transfusion

The first demonstration of the practical importance in clinical medicine of the discovery of the Rh factor was made by Wiener and Peters who described four cases in which haemolytic reactions occurred after the repeated transfusions of blood compatible by group. They were able to show that the sera of three of these

patients contained atypical agglutinins which gave reactions that were identical with the anti-rhesus serum and they showed that these three patients were Rh negative and that the blood responsible for the haemolytic transfusion reactions was Rh positive. In reporting ten further cases soon afterwards Wiener described the interesting phenomenon of "inapparent haemolysis." Following the transfusion of Rh positive blood to an Rh negative recipient already sensitized to the Rh agglutinin, there was sometimes no obvious haemolytic reaction in the form of haemoglobinuria and jaundice, but the recipient's haemoglobin value rose only temporarily after the transfusion and differential agglutination showed that the donor's erythrocytes had been rapidly eliminated from the recipient's circulation. If Rh negative erythrocytes were transfused a sustained rise in the haemoglobin value followed and the transfused erythrocytes survived normally. Wiener stressed the importance of watching for the phenomenon of inapparent haemolysis since if it were overlooked and a further transfusion of Rh positive blood given, a violent and obvious haemolytic reaction might follow.

The diagnosis of haemolytic transfusion reactions due to Rh incompatibility is not always straightforward. Samples taken from the patient soon after transfusion may contain some surviving Rh positive cells mixed with the patient's own Rh negative cells, and unless the observer is experienced he may falsely conclude that the patient is Rh positive. Further in examining the recipient's serum immediately after a suspected incompatible transfusion due to Rh differences, anti-Rh agglutinins may not be detectable, for they may be temporarily suppressed. In such cases the patient's serum should be titrated repeatedly during the ten days following transfusion and agglutinins may later appear.

Isoimmunization of Pregnancy and Relation to Haemolytic Disease of the Foetus

The formation of anti-Rh agglutinins, by Rh negative persons of either sex, as a response to the transfusion of Rh positive blood, is a comparatively uncommon event. Far more frequent is the formation of anti-Rh agglutinins by Rh negative women during pregnancy, as a result of stimulation by an Rh positive infant *in utero*.

Stimulation of maternal iso-agglutinins by foetal antigens was first demonstrated in 1905 by Dienst, who found an increase in

taking four hours or longer. The transfusion should be given as early as possible and a subsequent fall of the haemoglobin level to below 70 per cent. is an indication to repeat the transfusion. The technique of using a small cannula tied into the internal saphenous vein at the ankle has been found satisfactory, although the use of the umbilical vein in newly born infants seems worth further trial and the giving of transfusions into the tibial marrow is a useful resort when the intravenous route proves impracticable (see p. 275).

Anti-Rh Agglutinins in Milk : It is now well established that when anti-Rh agglutinins are present in the serum they may also be found, though usually in lower titre, in the milk and some authors have for this reason advocated that affected infants should be taken off the breast. However it is by no means certain that anti-Rh agglutinins can be taken from the gut into the serum in sufficient amount to cause any additional damage to the infant and the available evidence does not seem to justify this advice.

Physiological Jaundice : There is no evidence that physiological jaundice of the new-born is due to incompatibility between the mother's serum and the infant's erythrocytes, due either to Rh or ABO group differences. Serological tests are useful in detecting mild cases of "icterus gravis neonatorum" that might otherwise be overlooked and diagnosed as physiological jaundice.

Stillbirths and Miscarriages : Clinicians have long recognized that mothers who give birth to infants affected with one of the well recognized forms of haemolytic disease often also give birth prematurely to stillborn infants which may be oedematous, macerated or outwardly normal. Nevertheless there is evidence that only a small proportion of abortions and stillbirths, taking them as a whole, are due to iso-immunization to the Rh factor. For instance in a series of 70 mothers whose histories included stillbirths or repeated miscarriages, the writer and his co-workers found evidence of sensitization to the Rh factor in only 11. Moreover in 5 of these 11 a clinical diagnosis of hydrops foetalis had been made, so that only 6 out of 65 cases are left in which stillbirths or miscarriages, not clinically suspected to be related to haemolytic disease, may have been so. In a similar series of habitual abortions or inexplicable intrauterine deaths at full term Broman (1944) found evidence of Rh sensitization in two out of 17 instances.

Cases of Haemolytic Disease in which the Mother is Rh Positive : All observers have found that a small percentage of mothers of affected infants are Rh positive. At first it seemed that in these cases the Rh factor could not be concerned since an Rh

positive woman would not be expected to form anti-Rh agglutinins. Soon, however, Levine and his co-workers reported that they had found in the serum of an Rh positive woman an agglutinin against all Rh negative and some Rh positive bloods. Because of its ability to clump Rh negative bloods they called this agglutinin anti-Hr (Rh reversed). Other agglutinins related to anti-Rh may be formed in Rh positive persons. This is because there are subgroups of Rh and a person whose erythrocytes contain one type of antigen may form agglutinins against another type (*cf.* the rare formation of immune α_1 agglutinins by persons of subgroup A_2 following stimulation by blood of subgroup A_1). This preliminary description is given here to explain how even an Rh positive mother may form agglutinins related to anti-Rh and why it is therefore not necessary to postulate immunization against other blood group factors to explain the cases of haemolytic disease of the foetus in which the mother is Rh positive. The question of the rôle of ABO group differences between mother and foetus in causing foetal morbidity has frequently been raised in the past; there is some evidence that anti-A or anti-B agglutinins may occasionally play the same rôle as anti-Rh and cause haemolytic disease of the foetus, but the cases so far described are suggestive rather than conclusive.

Dangers of Transfusing Rh Negative Women with Rh Positive Blood: It is clear that the danger of haemolytic transfusion reactions from Rh incompatibility is greatest in pregnant or recently delivered women. When the foetus or infant is dead or obviously diseased the occurrence of haemolytic disease should be suspected; in other circumstances the infant may be diseased without arousing suspicion, *e.g.* the case of an infant alive *in utero* which after delivery will prove to be affected with icterus gravis. In such cases the risk from transfusing the mother is just as great, but there is no danger sign. Because of the occurrence of such cases it is desirable to test all women as soon as they become pregnant so that the Rh negative ones may be detected. Such women must never be transfused with Rh positive blood and the possibility that in the present or a subsequent pregnancy they will give birth to an infant affected with haemolytic disease must be kept constantly in mind. When a donor is wanted in an emergency for such women, it must be remembered that the husband's blood will never be suitable, since it contains the factor which has been passed on to the foetus and has stimulated the mother to produce opposing agglutinins. Some tragic cases have been recorded in which transfusion

of the husband's blood has been followed by a fatal haemolytic reaction.

So far attention has chiefly been paid to the dangers of Rh positive blood transfusions to certain women. A different aspect to consider is the danger to possible future infants caused by sensitizing a woman to the Rh agglutininogen by Rh positive transfusions. For instance Diamond (1942) has reported a case in which an Rh negative girl received many transfusions at the age of 17. Later she married and became pregnant and her first infant was affected with hydrops foetalis. This, the severest form of haemolytic disease, is so rare in first pregnancies that there is a strong suspicion that in this case it was attributable to sensitization of the woman by her previous transfusions. Whether this particular case is found convincing or not the possibility of an increased risk to the foetus being produced in this way obviously exists. Thus the question must be considered if any female who has not yet reached the climacteric should be transfused except after determining her Rh type and selecting suitable blood. As a first step this can simply be interpreted as detecting Rh negative women and not transfusing them except with Rh negative blood. However, recent knowledge of the occasional occurrence of immunization in Rh positive women due to subgroup differences (see p. 260) makes it likely that some kind of selection according to Rh subgroup will eventually have to be practised.

Rh SUBGROUPS

From the first it has been recognized that there are different subgroups of Rh and different kinds of anti-Rh agglutinins. The system is complicated and made particularly difficult to memorise by the fact that there are many different subtypes but only a few different antigens, the same few antigens occurring in different combinations in the different subtypes. In addition two different kinds of anti-Rh agglutinin are often present in the same serum. The discovery of the whole complicated scheme has been made particularly awkward to follow because the terminology has been changed several times. At present two main terminologies are in use. In Wiener's scheme symbols such as Rh_1 and Rh' are used to denote the different genes and the agglutinins are called anti- Rh_1 , anti- Rh' , etc. The chief disadvantage of this scheme is that the genes and agglutinins so named do not exactly correspond with one another, because the gene is a complex of antigens, whereas the agglutinin only reacts with a single antigen. The only way in which

the scheme can be made logical is to give separate names to the antigens and their corresponding agglutinins. This is done in Fisher's terminology and in it each gene is named according to its antigenic composition. Each Rh gene represents three antigens, one derived from each of the following three pairs: C c, D d, and E e. There are thus eight possible combinations (see Table), but in practice only three of these occur commonly, namely CDe (R_1), cDE (R_2) and cde (r) (R is used for Rh for the sake of brevity). A gene is inherited from each parent and there are thus six common genotypes or subgroups R_1R_1 , R_2R_2 , R_1R_2 , R_1r , R_2r , rr. The first five are all called Rh positive and subgroup rr is called Rh negative.

Corresponding to the six elementary antigens C c, D d, E and e are six agglutinins anti-C, anti-c, etc. Most (about 95 per cent.) human anti-Rh sera contain anti-D, either alone (60 per cent.) or in combination with anti-C (30 per cent.) or with anti-E (5 per cent.). The reason for this is easy to follow. When anti-Rh agglutinins are formed during pregnancy, usually the mother is Rh negative and the father Rh positive, belonging to one of the five common Rh positive subgroups described above. The foetus thus inherits R_1 (CDe) or R_2 (cDE) from the father and the mother (cde) is stimulated by the D antigen, in combination with C or E, to form anti-D with anti-C or with anti-E. Wiener uses the term anti-Rh₁ serum to describe the combination anti-C + anti-D, and anti-Rh₂ serum for anti-D + anti-E.

D appears to be a better antigen than C or E because anti-D is often found alone in a serum which on theoretical grounds is expected to contain anti-C or anti-E as well. Anti-C and anti-E agglutinins occasionally occur by themselves and a few examples of anti-c have been described. The latter are important because they agglutinate all Rh negative (cde) bloods and all the common Rh positive subgroups except R_1R_1 (CDe CDe). Only one example of anti-e has so far been reported and anti-d has not been encountered.

About 7 per cent of bloods contain the rarer genes R' , R'' , R_0 and R_z . The importance of R' (Cde) and R'' (cdE) is that they lack the D antigen and thus may fail to agglutinate when tested with anti-Rh sera, many of which, as described above, contain anti-D only. Thus such bloods may be classified as Rh negative and cause haemolytic reactions if transfused to a person whose serum contains anti-C or anti-E respectively. Furthermore, women of these types (Cde and cdE) will be classified as "Rh positive" if tested with a

diluted so that they no longer react with Rh negative cells. Another disadvantage is that animal sera agglutinate all infants' erythrocytes, irrespective of Rh type.

The use of human sera also has some disadvantages. For instance only those persons who have been immunized to the Rh antigen by transfusion or pregnancy have anti-Rh in their sera and only about one-tenth of these will have anti-Rh in sufficient strength to make satisfactory testing reagents. On the other hand a laboratory that is engaged in testing large numbers of sera suspected of containing anti-Rh agglutinins will have no difficulty in finding suitable sera and it is then usually feasible to take 400-500 cc. of blood and thus obtain about 200 cc. of satisfactory serum. If this contains anti-A and anti-B agglutinins these can be removed by absorption at 5° C. with group A and B Rh negative cells, or alternatively they can be neutralized by the addition of purified group specific substances or of saliva from group A and B secretors. If the sera are merely to be used for testing group O donors to discover those who are Rh negative, it will not be necessary to remove the anti-A and anti-B agglutinins. The sera should be Seitz-filtered and then stored in small ampoules, frozen solid.

Testing Unknown Cells: The procedure followed in testing unknown cells will vary according to the purpose of the test and the test sera available. If the purpose is to select an Rh negative donor for transfusion there is no point in adopting methods to reveal the genotypes of the Rh positive bloods, and it is simply necessary to ensure that bloods of the genotype rr are selected. This can be achieved by using both anti-Rh' and anti-Rh₂ sera (or anti-Rh₁ and anti-Rh' ; all three agglutinins anti-C, anti-D and anti-E must be present in the test sera) In practice the appropriate sera may not be available, in such cases anti-Rh₁ serum may be used alone with very little risk since it reacts with all Rh positive bloods except the very rare R"r and R"R" genotypes. Furthermore if the recipient's serum contains anti-Rh agglutinins these are most likely to be anti-C + anti-D or anti-D alone and will in that case not react with R" bloods. Thus until satisfactory stocks of the different types of anti-Rh serum are available one may have to use anti-Rh₁ (anti-C + anti-D) alone.

In the rare cases where a potentially incompatible blood is selected by this compromise method it is still likely that this fact will be revealed by the direct compatibility test.

If on the other hand it is desired to determine the Rh genotype of the blood, anti-C, anti-D, anti-E and anti-c sera

are all needed, and even these will not suffice for the exact definition of all the genotypes

Technique: One small volume (say 0.04 cc.) of a 2 per cent. suspension in citrate of the blood to be tested is mixed with an equal volume of test serum in a small glass test-tube (approx. 3×0.7 cm.) The tube is left in the incubator at 37°C . for 2 hours. At the end of that time the sediment is drawn up gently into the stem of a Pasteur pipette and gently delivered and spread on to a glass slide. The preparation is examined under the low power of the microscope for the presence of agglutination. Appropriate controls must be included.

A tube technique is essential for the detection of anti-Rh agglutinins; if carried out directly on slides usually no agglutination is produced. According to Wiener and his co-workers exceptional anti-Rh sera give good clumping by the common slide technique and this may be associated with the wide temperature range of action of these particular sera. This is only true when using weak cell suspensions; Diamond *et al.* (1945) state that if Rh positive whole blood (0.2 cc., oxalated) is mixed with anti-Rh serum (0.1 cc.) on a slide, good reactions develop even when the serum contains weak anti-Rh agglutinins or "blocking" antibodies (see below). In view of the difficulties inherent in slide testing this method is likely to supplement rather than supplant the tube technique.

The weakness of most Rh agglutination reactions goes far to explain the delay in discovering the occurrence of anti-Rh agglutinins in human sera. It has been suggested that this weakness is due to the fact that compared with the A and B groups there are far fewer Rh haptene groups per erythrocyte. Moreover when "blocking" antibodies are present the number of sites available on the erythrocyte surface for the attachment of anti-Rh agglutinins is further reduced.

Testing Unknown Sera: All sera so far described react with either Rh₁, Rh₂ or rh cells or with some combination of these three types. Thus if it is simply desired to prove or disprove the presence of anti-Rh agglutinins of some type in a given serum it is sufficient to test it against cells of genotypes R₁R₂ and rr and the technique will be the same as that described above. If exact identification of the agglutinins is desired it will be necessary to include bloods of types Rh' and Rh'' as well.

"Incomplete" or "Blocking" Antibodies: Race (1944) and Wiener (1944a) have independently described an interesting phenomenon of practical importance in Rh testing. Some sera contain anti-Rh antibodies which are capable of combining with

Rh antigens but not of causing agglutination. Although they do not cause agglutination *in vitro* they can give rise to haemolytic disease of the foetus and presumably haemolytic transfusion reactions. Their presence in a serum can be demonstrated by suspending Rh positive cells in the serum and then testing the same cells with a serum containing ordinary anti-Rh agglutinins. When the expected agglutination fails to occur in the second stage of the test it may be presumed that the Rh antigen has been blocked by "incomplete" antibodies in the first serum.

Wiener (1944c) advocates the following technique: "First, one drop of a 2 per cent. suspension of Rh positive cells and a drop of the patient's serum are mixed in a small test-tube and allowed to react in a water-bath at 38° C for 30 to 60 minutes. Then a drop of a suitable dilution of an active anti-Rh serum is added and after an additional incubation period of 30-60 minutes, the reactions are read. If blocking antibodies are present, no agglutination will occur, or the clumping will be markedly weakened."

Anti-Rh agglutinins and blocking antibodies frequently occur together in the same serum and this leads to anomalous results; depending on different concentrations of cell suspension and different dilutions of serum, agglutination may or may not occur.

Both Race and Wiener report that the blocking antibodies are specific and so far (with one exception) they have only encountered antibodies of the specificity of anti-Rh₀ (anti-D).

Race and Taylor have described some cases in which the history sounded typical of haemolytic disease and yet in which no anti-Rh agglutinins had been detected. In some of these they succeeded in demonstrating blocking antibodies in the mother's serum.

In view of these findings the examination of a serum cannot be considered complete until blocking antibodies have been sought; however this will only be necessary when immune anti-Rh agglutinins cannot be demonstrated.

Coombs *et al.* (1945) have described a test which shows that when red cells react with blocking antibodies, globulin becomes absorbed on to their surface. The presence of the adsorbed globulin can be demonstrated by testing the cells against a rabbit serum prepared by injecting human globulin (or serum) into rabbits, i.e. an anti-human-globulin serum.

A complete examination of a serum for evidence of Rh immune bodies should be made in two stages. Firstly the serum is tested against a suspension of Rh positive cells so as to examine for agglutinins in the ordinary way. If agglutination does not occur one of

the following two tests is applied : either a drop of a potent anti-Rh serum is added (to see whether or not the red cells have been acted upon by blocking antibodies) ; or the red cells are washed and then tested against the rabbit "anti-human-globulin" serum (also to see if the red cells have taken up blocking antibody).

The anti-human-globulin test has proved very useful in the diagnosis of haemolytic disease of the newborn. A few drops of blood are taken from the infant into citrate-saline ; the red cells are then washed well and tested against the rabbit serum. Agglutination of the infant's red cells can be taken as diagnostic of haemolytic disease.

Compatibility Tests : Many sera contain only weak anti-Rh agglutinins and many also contain blocking antibodies. Therefore a simple direct matching test is not very reliable to guard against the danger of haemolytic transfusion reactions due to Rh incompatibility. At present there is no general agreement as to which is the most reliable method of detecting sensitisation to Rh but it is likely that the "anti-human-globulin test" will prove at least as dependable as any other. The donor's red cells, after incubation with the recipient's serum, are examined for evidence of agglutination ; if none is seen the red cells are well washed and tested against the anti-human-globulin serum. Agglutination in this stage of the test shows that the red cells have absorbed a blocking antibody from the recipient's serum and that transfusion is dangerous. If, however, the erythrocytes remain unagglutinated in both stages of the test, the blood can safely be transfused.

The most important single precaution in preventing Rh incompatibility in transfusion is to exercise great care in the selection of Rh negative donors. At the same time direct matching of the donor's erythrocytes against the recipient's serum should be practised as a routine to ensure that blood of compatible ABO group is given and to detect any rare incompatibility not guarded against by the form of donor selection employed (see p. 284).

When time is very short a quicker but less sensitive technique for the direct matching test may be used : instead of waiting for 2 hours the tubes may be removed from the incubator after 30 minutes, centrifuged at low speed for 1 minute and then examined for agglutination.

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References in this chapter have been reduced to a minimum and many sources of information have not been directly acknowledged. For a useful guide to the literature the reader is referred to a recent review by BOYD, W. C. *Arch Path* 40. (1945) 114.

CHAPTER XXV

THE TRANSFUSION OF BLOOD AND BLOOD PRODUCTS

Selection of a Donor

ANY adult normal individual is suitable; usual age limits are 18-65. It is preferable that a donor should not have had a heavy meal within 2 to fainting. or above. Gc

Syphilis and malaria form the two significant transmissible diseases that must be excluded in whole blood transfusion. In the case of the latter it is safe to use a donor with a past history of malaria provided he has not had a febrile attack that could be attributed to it during the preceding 5 years, nor resided during that period in a malarial district. Individuals who are allergic, or who have had "catarrhal" or infective jaundice during the previous 12 months are best avoided.

The donor should be of the same blood group as the patient; the use of group O as "universal donor" for recipients other than group O, although warrantable under emergency conditions, carries some risk of damage to the recipient's red cells, particularly in large volume transfusions.

Blood from an Rh negative donor of compatible A, B, O group is required for transfusion of cases in which immunization to the Rh factor is suspected or proved (see p. 268).

In addition to the routine grouping a direct matching test for compatibility between the patient's serum and the red cells of the donor or bottle of stored blood it is proposed to use, should always, if possible, be carried out (see p. 284).

A donor (male or female) in normal health can safely give 400-600 cc. of blood at minimal intervals of 3 months.

Collection of Blood

The Medical Research Council Blood Transfusion Outfit consisting of standardized components has superseded the miscellaneous methods previously employed. The M.R.C. bottle (Fig. 11) is slightly waisted to facilitate holding, fitted with an aluminium screw cap with a 4 mm. rubber wad, and provided with a metal band and loop

at the base for hanging the bottle in the inverted position. The bottle is marked at 540 cc. and 180 cc., the latter mark because the volume of anti-coagulant originally used was 180 cc.

Anti-coagulant : The anti-coagulant recommended is 120 cc. of a 2 per cent. solution in freshly distilled water of disodium hydrogen citrate and 2.5 per cent glucose; this is satisfactory for 420 cc. of fresh or stored blood. For fresh blood transfusion the anti-coagulant may consist merely of 120 cc. of a 2 per cent. solution of tribasic sodium citrate.

Sterilization of bottles and contained anti-coagulant solution is carried out by autoclaving at 20 lb. pressure for 30 minutes; with the disodium salt no significant caramelization of the glucose results. Autoclaving of the anti-coagulant solution should be carried out within 24 hours of its preparation.

Collection of Blood : The standard collecting set is put up and sterilized by autoclaving in individual packets of unbleached calico or wrapped in cellophane paper in tins. Two patterns of apparatus for blood collection are in common use, depending on whether the cap of the collection bottle is replaced by a rubber bung and glass tubing (Type A) or perforated by two needles (Type B).

Type A. Replacement of the cap by a rubber bung and glass tubing (Fig. 11) : The screw cap is removed and placed between the folds of a sterile towel. The bottle is then fitted with a sterile "taking set" comprising a rubber bung pierced by two 3 inch (7.5 cm.) glass tubes (6-7 mm. external diameter), one of which is lightly plugged with cotton wool and serves as an air outlet. To the other, which projects slightly further through the bung, is attached about 14 inches (35 cm.) of rubber tubing and a carefully sharpened stainless steel needle (see p. 278) which is protected with a small glass test-tube. A short length of glass tubing is inserted about 2 inches (5 cm.) above the needle to serve as a window so that the operator can observe the passage of blood and obtain rapid confirmation that the vein has been entered.

The donor lies flat on a bed or couch, the head being supported by a pillow. Blood is collected from a vein in the antecubital fossa the elbow resting on a firm arm cushion. A sphygmomanometer cuff or tourniquet is applied to the upper arm to produce venous stasis and the skin over the selected vein cleaned with suitable antiseptic; approximately 0.1 cc. of a local anaesthetic—2 per cent. procaine hydrochloride—is injected intradermally over the selected vein. The small glass tube protecting the needle of the taking set is removed and the needle inserted into the vein and held

in position whilst the bottle fills. Gravity is usually sufficient to maintain a steady flow of blood. When the bottle has filled to the 540 cc. mark the tourniquet is released and the needle withdrawn from the vein; the blood remaining in the rubber tubing is run

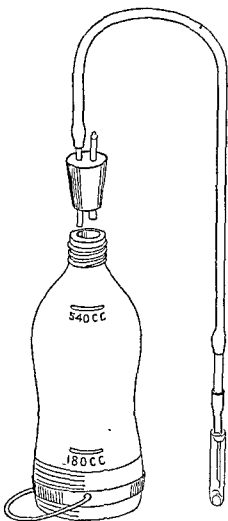


FIG. 11.

FIG. 11.—Standard M R C. blood bottle and collecting set (Type A)

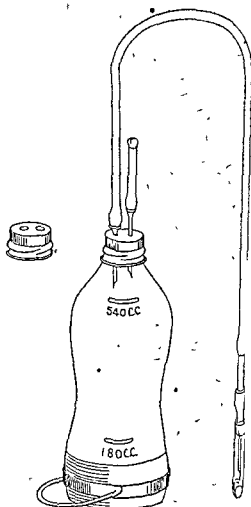


FIG. 12

FIG. 12.—Standard M R C. blood bottle and collecting set (Type B).

into a sample tube for confirmatory grouping and a Wassermann or Kahn test. The rubber bung with its tubing is removed and the aluminium cap reapplied with sterile precautions.

Type B. Perforation of the cap by two needles (Fig. 12): In this method the aluminium cap of the bottle is perforated by two holes

3 mm. in diameter; the "taking set" instead of the rubber bung and glass tubing of Type A has two needles, one of which is attached to the rubber tubing and vein needle, the other carrying a small length of rubber tubing plugged with cotton wool serves as an air outlet. Collection of blood is carried out as described above and just prior to entering the vein the two needles are inserted into the bottle by perforating the rubber wad which shows through the holes of the aluminium cap.

The donor should continue to lie flat for at least 10 minutes after the blood withdrawal and before being given any light refreshment. Smoking is not advised for half an hour. A small dry dressing is all that is required for the arm.

Infusion of Blood: The standard blood giving apparatus (Fig. 13) consists of a rubber bung pierced by two pieces of glass tubing, one $9\frac{1}{2}$ inches (25 cm.) long reaches almost to the bottom of the bottle and serves as an air inlet; its outer end is fitted with a small cork. The other tube $2\frac{1}{2}$ inches (6.5 cm.) long, has attached in sequence to its outer end 6 inches (15 cm.) of rubber tubing, a drip feed bulb,

FIG 13—Standard MRC bottle and administration set (A) with gas mantle filter, (B) with metal gauze filter.

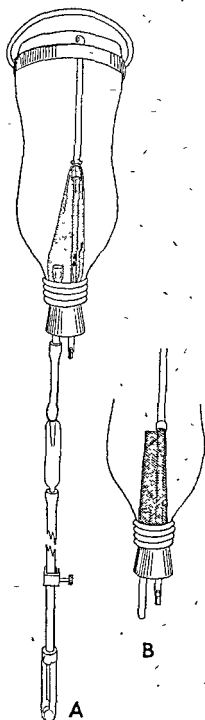


FIG 14—Standard metal cannula.

and 1 yard (1 m.) of rubber tubing terminating in the standard narrow bore needle used for the giving of blood (see p. 278). The terminal 2 inches (5 cm) of tubing attached to the needle is detachable by means of an adaptor fitting. A short distance above this is a screw clip by which the flow of blood through the set as denoted by the rate of flow in the drip bulb can be controlled. A metal cannula (Fig. 14) can be attached directly to the male adaptor after removal of the needle and needle tubing when it is necessary to cut down on the vein.

To prevent clots entering the set one of two types of filter is employed to cover the inner end of the small outlet in the rubber bung:

(a) Gas mantle filter. This is an open cylindrical stocking of finely knitted cotton as used in the manufacture of gas mantles. It is $3\frac{1}{2}$ inches (9 cm.) long, threaded with a purse string at each end and tied at one end to the rubber bung and at the other to the air inlet glass tube (Fig. 13 A).

(b) Metal gauze filter. This consists of a metal gauze cylinder approximately $2\frac{1}{2}$ inches (6.5 cm.) in length and $\frac{3}{8}$ inch (1.5 cm) in diameter. One end is open and the other partially closed by a fold of the gauze. The filter is slipped on the air inlet glass tube, open end towards the bung, and slid up the tube till it makes contact with the narrow end of the bung, thereby enclosing the inner portion of the outlet tube. As a precaution against displacement a $\frac{1}{4}$ inch (0.5 cm.) piece of pressure tubing is slipped on the glass tube immediately beyond the filter (Fig. 13 B).

The standard giving set is packed in an unbleached calico bag or in cellophane paper in a tin and sterilized by autoclaving.

To give blood invert the bottle two or three times to secure even suspension of red cells; stand the bottle for 20–30 minutes in water at 37° C. to warm the blood (overheating must be carefully avoided). The aluminium cap is next unscrewed and removed and the rubber bung of the giving unit inserted into the mouth of the bottle. The screw clip on the rubber tubing is adjusted to close its lumen and the bottle hung up by means of the metal band and loop so that the apparatus is suspended vertically above the vein to be used. Disconnect the needle and short piece of rubber tubing from the rest of the giving unit by means of the adaptor and place them in the folds of a sterile towel. Remove the cork from the outer end of the air inlet tube and unscrew the screw clip, holding the male adaptor just below the level of the drip bulb. Blood will now flow through the apparatus dispelling all the air. When this has been

done the tubing can be clamped by means of artery forceps or by the screw clip.

A vein in the antecubital fossa is usually chosen for the transfusion, the elbow being fixed with a back splint if the patient is restless. The overlying skin is cleaned with suitable antiseptic and the vein rendered full and prominent by means of a sphygmomanometer cuff or a tourniquet. The needle with the short piece of rubber tubing attached is inserted into the vein, a satisfactory insertion being indicated by an efflux of blood from the female adaptor; as soon as this occurs the pressure round the upper arm is released and the male adaptor on the suspended unit joined to the female adaptor; the rate of blood flow is adjusted by means of the screw clip. Fixation of the needle in the vein is ensured by strapping its shoulder and attached tubing to the skin of the patient's arm.

Dosage and Rate of Flow : Dosage can only be decided individually for each case. In children it is somewhat higher in proportion to body weight than in adults and for them 15 cc. per kg. (2½ lb.) body weight is an approximate guide. The anticipated rise in haemoglobin resulting from the transfusion of one bottle of whole blood (with the usual proportion of anti-coagulant solution) is approximately 8 per cent. (Haldane) in an adult in the absence of simultaneous blood loss or blood dilution. When only 1-2 bottles of blood are to be administered the blood can be given at a fast drip rate (one bottle per half hour or less) in the absence of cardiac or circulatory embarrassment. In volumes above this (including plasma and serum) the rate of flow should be reduced to 40 drops per minute through the drip bulb (approximately 1 bottle per 4 hours).

Choice of Vein : A vein in the left or right antecubital fossa is usually the most prominent. If the transfusion is likely to take several hours, a vein in the forearm is rather more satisfactory as the patient can be permitted greater freedom of movement at the elbow. In infants and young children and in adults whose arms have been injured, the internal saphenous vein situated just in front of the internal malleolus is recommended; for this vein the insertion of a cannula is recommended. In very young infants a scalp vein may be employed.

Intramedullary Administration : In rare cases, e.g. extensive severe burns or scalds, where no surface veins are available, fluids may be administered into the marrow cavity. In adults and in children over 2 years the sternum is the most convenient site; in

infants the tibia may be used, although the risk of sepsis here appears greater. A Salah Sternal puncture needle, with winged guard (Fig. 15) is used and may be inserted through burned or scalded areas.

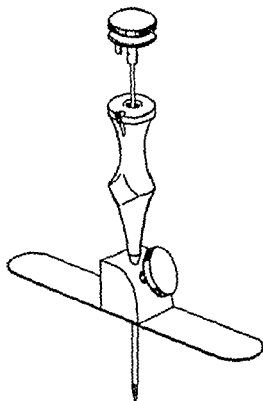


FIG. 15.—Salah sternal puncture needle with winged guard

Strict surgical asepsis must be adhered to. In the case of the sternum the manubrium is the best site, in the tibia the site of puncture is the antero-medial surface at the level of the tibial tubercle. The procedure followed is as for diagnostic sternal puncture (see p. 243), the needle when inserted being connected with the male adaptor of the giving unit, prepared as for an ordinary intravenous transfusion. Gravity usually suffices for a satisfactory flow; transfusion into the tibial marrow tends to be slow at first, but subsequently to increase in rate.

Stored Blood, Blood Products and Concentrated Red Cell Suspension

Stored Blood: Blood collected with sterile precautions into the glucose disodium hydrogen citrate solution previously described may, if kept at $2-4^{\circ}\text{C}$, be safely used up to 21 days from the date of collection, for raising the haemoglobin level and restoring blood volume. Blood stored for more than a few days rapidly deteriorates as regards functional leucocyte content, platelets and other clotting factors and also immune bodies, specific and non specific. Fresh blood is therefore indicated in agranulocytosis, thrombocytopenia, haemophilia and other haemorrhagic diatheses, and in severe infections. The fragility of the red cells in stored blood gradually increases, especially during the third week; fresh blood is consequently preferable in the treatment of a blood disease where maximum durability of the transfused cells is required.

A bottle of stored blood should be carefully inspected before use; if there is haemolysis in the supernatant plasma or if there is any pigment change in the sedimented red cells the bottle should be discarded.

Blood Products (human plasma and serum): Plasma or serum are made available in the liquid or dried state. In their preparation the serum or plasma from bloods of various groups are pooled; this results in a product low in agglutinin content which can be safely used for a patient of any group. Liquid or dried serum or plasma need not be refrigerated but should be stored in a dark cool place. The liquid product (except that which has been reconstituted) is not entirely stable and should not be given if it shows turbidity, deposit or extensive clot formation.

Concentrated Red Cell Suspension: This is prepared by taking two standard bottles of whole citrated blood of the same group, in which maximum sedimentation of the red cells has occurred (usually 5-6 days after date of collection). The supernatant plasma in each is syphoned off and the red cell deposit in one bottle added to that in the other, the mixture being topped up to the 540 cc. mark with a small volume of saline preferably hypertonic (1.1 per cent.). Sterile precautions are maintained throughout and it is advisable that the suspension be used within a few hours of the preparation. Such concentrated suspension is particularly useful when it is desired to raise the haemoglobin and red cell count rapidly with minimal increase of blood volume—e.g. in aplastic anaemia, anaemia prior to operation, anaemia associated with heart disease, or with nephritis accompanied by oedema. Anticipated haemoglobin increase per bottle when given to an adult is 15 per cent. (Haldane) compared with 8 per cent. with whole blood. The reaction incidence is probably lower than with whole blood, serum or plasma.

Transfusion Reactions

These may be immediate, delayed for a few hours or days, or delayed for several weeks; they may be haemolytic or non-haemolytic.

Immediate post-transfusion (non-haemolytic) reaction is best controlled by the hypodermic injection of morphine tartrate 10-20 mg. and adrenaline hydrochloride (1/1000) 0.5-1 cc., the dose varying according to the size of the patient and the degree of reaction. In recipients in whose serum cold agglutinins have been demonstrated, special care must be taken to maintain the transfused

blood at body temperature throughout the operation; such cases are likely to react to cool blood. Development of loin pain may be an early sign of haemolytic reaction; if it occurs during a transfusion this should be terminated until the possibility of incompatibility has been re-examined and excluded. In all cases the few residual cc. of blood, plasma or serum remaining in the bottle at the end of a transfusion should be preserved for 24 hours in a refrigerator or cool place, in case a sample may subsequently be required for laboratory investigations.

Delayed reactions as a rule present themselves in the form of jaundice; when this is due to lysis of the red cells in the patient's circulation it appears from a few hours to a few days after the transfusion of blood. When the jaundice appears several weeks after the administration of blood, plasma or serum it is due to an icterogenic agent contained in the latter (and derived from a donor) producing hepatitis of varying degrees of severity; the incubation period of this type of hepatitis is between 60 and 90 days. The batch number of plasma or serum used should be recorded in order that icterogenic batches may be detected and withdrawn as soon as possible.

Appendix

NEEDLES: For taking blood—stainless steel, size 2.4 mm. diameter by 35 mm. long, olive mounts, "Record" fitting, short bevel point, polished inside and out.

For giving blood—stainless steel, size 1.5 mm. diameter by 35 mm. long, olive mounts, "Record" fitting, short bevel point, polished inside and out.

It is imperative that all needles for venepuncture be sharp and the point of each should be scrutinized with a hand lens before incorporating in the collecting or giving set. Important criteria are a short sharp straight point, gradual sloping bevel, absence of shoulder and absence of burr. Care should be taken to preserve the point by replacing the small glass tube needle cover immediately after use. Attention to these details facilitates transfusion both as regards operator, donor and patient.

CLEANING OF APPARATUS: It is essential that all parts of the apparatus employed be scrupulously clean. Thorough rinsing immediately after use with cold tap water or a weak soda solution facilitates subsequent cleaning after dismantling.

BOTTLES are thoroughly brushed inside, using warm tap water. They are then boiled for half an hour in a weak solution of washing soda (1.25 per cent) and rinsed in 5 per cent. hydrochloric acid. After

a further washing first with tap and finally (if possible) with distilled water they are allowed to drain and dry. — Soapy water may be used if washing soda and hydrochloric acid are not available.

GLASS TUBING, DRIP BULBS are washed through with tap water, then treated with weak soda and acid solutions as the bottles and finally rinsed with tap water and distilled water and dried in an oven.

RUBBER-TUBING is treated as for glass tubing, except that the lengths are drained and dried by hanging vertically in a warm place. An elongated thin brush on the end of a long flexible piece of wire or a thin rifle brush are especially suitable for cleaning the interior of rubber tubing.

RUBBER WADS, RUBBER BUNGS are washed in hot soapy water, the wads being removed from the caps. They are then rinsed in tap, followed (if possible) by distilled, water, and dried.

NEEDLES are washed through with tap water under pressure and cleaned with a stilette; for the butt, cotton wool on the end of a swab stick is used. They are then rinsed in distilled water, transferred to 70 per cent. spirit and subsequently dried. Special care is taken to avoid blunting the points and the bowls in which the needles are placed are lined with lint. Adaptors are dealt with in similar fashion.

FILTERS. Gas mantle type, owing to staining with blood, are best discarded after use.

Metal gauze type are rinsed and boiled in tap water. They are then well brushed with a small firm fine-bristle brush in running tap water, rinsed in distilled water and dried in an oven.

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CHAPTER XXVI

BLOOD GROUPING

The A B O Blood Groups and Principles of Recognition: Two agglutinogens—A and B—are present in human red cells; they may occur either together, or singly, or be absent. Corresponding to them, human sera contain two agglutinins—alpha or anti-A and beta or anti-B. Contact between an agglutinin and its homologous agglutinin will result in haemagglutination. The formulae of the blood groups is as indicated in Table I.

TABLE I

<i>Groups</i>	<i>Agglutininogen in red cells</i>	<i>Agglutinins in serum</i>
AB	A and B	neither
A	A	beta (anti-B)
B	B	alpha (anti-A)
O	neither	alpha and beta

The group of a blood is ascertained by testing the cells for agglutininogen content against known Group A and Group B sera containing the beta and alpha agglutinins respectively. Confirmation is obtained by testing the serum also for agglutinin content with known Group A and Group B cells

Grouping Serum. Sera for grouping must show :

- (1) High titre of the alpha and beta agglutinins;
- (2) Capacity to react with A₂ and A₂B cells;
- (3) Absence of cold agglutinins;
- (4) Absence of prozone phenomenon;
- (5) Absence of any tendency to cause rouleaux formation;
- (6) Freedom from fat,
- (7) Freedom from bacterial infection.

TITRE The minimal initial advisable titre of the alpha and beta agglutinins is 1:160 using the technique described below (Taylor *et al.*, 1942) by observing the serum and appropriate red cells on a glass slide.

Method of titrating test sera.—Take in a row 10 small test tubes (approx. 5 × 0.6 cm.); to the first add two standard drops (each approximately 0.05 c.c.) of 0.85 per cent. saline and to each of the remainder 5 drops. Eight drops of the serum to be tested are added

to the first tube, the contents mixed and 5 drops transferred to the second tube; the contents of the second tube are next mixed, 5 drops added to the third tube and the process repeated down to the tenth tube, the last 5 drops of the diluted serum being discarded. Five drops of a dilute suspension of cells (Group "A" for a Group "B" serum and vice versa)—approximately 5 per cent. of whole blood in 0.85 per cent saline—are added to each tube and mixed by gently shaking. The tubes are capped with cylindrical glass caps. Readings are recorded after standing for 2 hours at room temperature. Each tube is picked up and the cell deposit at the bottom of the tube disturbed by flicking with a finger. The contents of every tube which does not show definite agglutinates to the naked eye are examined microscopically by placing a drop of the mixed contents on a slide under the low power of the microscope. The strength of the reaction is designated as follows:

C (complete)	Clumps of cells in clear surrounding fluid, visible to naked eye
V (visual)	Rather weaker clumping, distinctly visible to naked eye, but with surrounding fluid not clear
+4	Large clumps under microscope.
+	Small clumps under microscope
±	More or less uniform clumps of 4-6 cells

The lowest dilution recording a \pm result is taken as the end point. The dilution in the tubes corresponds to 1 in $2\frac{1}{2}$, 1 in 5, 1 in 10, and so on to 1 in 1,280. A serum with a minimal alpha or beta agglutinin titre of 1 in 160 may be expected to maintain a satisfactory potency for at least six months.

AGGLUTINOGEN A_2 : There are two different varieties of Group A and Group AB blood depending on whether the A agglutinin is A_1 or A_2 . The importance of recognizing this subdivision of the A agglutinin is that the cells of A_2 and particularly A_2B bloods react more weakly than those of A_1 and A_1B with Group "B" (anti-A) test serum. If the latter is not specially selected the reaction may be so weak as to be undetected and the sub-groups A_2 and A_2B may be erroneously classified as Groups O and B respectively. Approximately one in five of Group A or Group AB individuals have the sub-group A_2 . It is important therefore that every Group "B" serum used for grouping purposes should have a satisfactory titre of agglutination for A_2 as well as A_1 cells, a minimum of 1 in 160 for both. Recognition of A_2 cells can most simply be obtained by testing individually cell suspensions (approximately 5 per cent of whole blood in normal saline) from batches of a dozen known Group A subjects against the serum of a

If the titre of a Group "B" is satisfactory for A₂ cells it will give the same or a higher figure for A₁ cells; the reverse is not true.

COLD AGGLUTININS: Some sera contain an appreciable titre of non-specific cold agglutinins. Such agglutinins cause interference if grouping is carried out at low temperatures. They can be removed to a considerable extent by allowing the serum to stand in contact with its clot and loose cells in the refrigerator (2°-4° C) overnight after gentle inversion. The serum is separated next morning whilst still cold.

PROZONE PHENOMENON: This is absence or weak agglutination in the lowest or lower dilutions of a serum which may otherwise have a high titre. The condition when present tends to disappear with storage of the serum or it may be more quickly eliminated by heating the serum to 55° C. for 20-30 minutes.

ROULEAUX FORMATION: This is recognized by testing the serum against a saline suspension of its own or compatible cells using the tile technique (see p. 283). Rouleaux formation is liable to suggest a weak positive agglutination reaction but the particles are small and of the same size; true agglutinates are variable in size.

FAT: Fat tends to accelerate deterioration of agglutinin titre; donors from whose blood grouping serum is to be prepared should have had no fatty food for 3 hours before being bled.

BACTERIAL INFECTION: Sterility must be maintained at all stages in the preparation of stock grouping serum. Infection may cause pan-agglutination, rouleaux formation or fall in titre. The use of antiseptics or preservatives is not advisable.

Storage of Grouping Sera: Grouping sera are preferably stored in 2 cc., 5 cc. or 10 cc. volumes in bottles two-thirds full, frozen at -10° C. Prior to use the sera are thawed by standing the bottle in warm water or an incubator and subsequently shaking to mix. After the requisite amount of serum is removed the bottles are replaced in the refrigerator and the serum refrozen. In the frozen state potency is preserved almost indefinitely. If freezing is not possible storage should be at 2-4° C. at which temperature the titre usually remains satisfactory for at least six months. Deterioration may be rapid if the serum is kept at room temperature and exposed to light. It is standard in Great Britain to use white glass for containers for Group "A" serum and amber for Group "B." A more recent development is the preservation of grouping serum in the dry state.

Determination of Blood Groups: Blood grouping results are most reliable when cells and serum are both examined. Either of two methods may be employed.

1. The Tube Technique: (a) Test for agglutinogens in the cells. Use tubes about 5 x 0.6 cm preferably without lips. For each test use pairs of tubes placed one in front of the other; for a number of

cell tests the tubes may be placed in rows. Cells to be tested are made up in emulsions of about 5 per cent with saline. To the first row of tubes add 2 drops of the known grouping serum "A" and to the second the same amount of serum "B"; test sera are diluted to one half with saline to avoid rouleaux formation or pseudo-agglutination. With the same pipette add 2 drops of the emulsion of red cells to be tested to each pair of tubes. Rinse the pipette carefully between taking up each cell sample. Put up controls of known A, B and O cells. Read as above under testing of titre of grouping serum (see p. 281).

(b) Test for agglutinins in serum. This is carried out as above except that emulsions of red cells of known groups are used in the pairs of tubes and the serum to be tested, diluted to one half with saline, is added in equal amount. The range of agglutinin titre in different sera varies considerably and in the case of the beta agglutinins of Group "A" serum may be very low or even absent. In infants the agglutinins may not develop until some months after birth.

2. The Tile Technique: The red cell emulsions are mixed with the known grouping sera in equal bulk on a white or opal glass tile or on transparent slides on a white background. In cold weather the tile should be gently warmed to guard against "cold" agglutination. The procedure is exactly the same for identification of the serum agglutinins. In both cases the drops of serum and cell emulsions should be mixed thoroughly with a glass rod. After 10-15 minutes standing the preparations are agitated by rotation of the tile. Agglutination is readily seen with the naked eye, but confirmation by hand lens is advisable.

Of the two techniques that of the tile is the less onerous and is reliable for clinical purposes.

With both methods in diagnosing cells as Group "B" care must be taken against fine agglutination with the Group "B" (anti-A) serum which would mean that the cells really belong to the sub-group A_2B . The examination of the serum for agglutinins is not necessarily a safe check in this case since as shown in Table 2 the serum from a number of A_2B individuals contains the agglutinin α_1 which reacts with A_1 but not A_2 cells. One of the criteria of a satisfactory Group "B" (anti-A) grouping serum is its capacity to react with A_2 cells. The most certain way of eliminating this error is to test the serum of a supposedly Group B individual with known A_2 cells; if agglutination occurs the serum must contain the agglutinin α_1 which reacts with A_2 as well as A_1 cells and the serum must be that of a Group B individual. The serum of an A_2 or A_2B person may each contain a small amount of α_1 agglutinin which will agglutinate A_1 cells. The presence of this agglutinin is confirmed by the absence of any reaction when A_2 cells are used.

A list of the usual and possible extra iso-agglutinins associated with the A, B, O groups is indicated in Table 2.

TABLE 2

Group and Sub-group.	Reacts with agglutinins	Agglutinins usually occurring in serum.	Agglutinins (possible extra).
A ₁	alpha ₁ and alpha	anti-B (beta)	anti-O(alpha ₁) (very rare) reacts with 95 % of A ₁ and all O cells
A ₂	alpha (always) anti-O (alpha ₂) (95 % of cases)	anti-B (beta)	alpha ₂ (1-2 %) reacts with A ₁ and A ₁ B cells.
A ₁ B	alpha ₁ , alpha, and beta	None	anti-O (alpha ₁) (very rare) reacts with 95 % of A ₁ and all O cells.
A ₂ B	alpha and beta; seldom if ever with anti-O	None	alpha ₂ (25-30 %) reacts with A ₁ and A ₁ B cells.
B	beta	anti-A (alpha and alpha ₁)	anti-O (alpha ₂) (very rare) reacts with 95 % of A ₂ and all O cells.
O	anti-O (alpha ₁)	anti-A (alpha and alpha ₁) anti-B (beta)	None.

Direct Matching: Direct matching between the patient's serum and the red cells of the donor or bottle of stored blood which it is pro-

patibility Either the tube or tile technique may be employed.

Errors in Direct Matching: (1) Pseudo-agglutination or rouleaux formation This is the most common cause of an apparent incompatibility between a serum and red cell suspension which should be compatible according to individual routine grouping tests. The phenomenon is particularly likely to be marked in the sera of patients with septic conditions and high fever; it is closely related to a high sedimentation rate. It is recognised by the fact that if the serum be diluted 1 in 2 or 1 in 3 with normal saline before the addition of the red cell suspension the phenomenon tends to disappear.

(2) Cold agglutinins In this condition the patient's serum will agglutinate at room temperature, red cells that should be compatible, (including his or her own red cells), owing to the presence of a cold agglutinin (usually non-specific) active at this temperature range. It may be detected by repeating the test at 37° C. when agglutination will no longer be apparent.

If an apparent incompatibility in the direct matching test can be explained by rouleaux formation or by cold agglutination, the trans-

fusion may safely proceed. If such an explanation is not forthcoming, the original grouping of the patient's blood and that of the donor or stored blood must be re-examined for error.

Rh Compatibility and Cross Matching (see p. 268).

H. F. BREWER.

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CHAPTER XXVII

THE SEDIMENTATION RATE OF THE RED CELLS

THE estimation of the sedimentation rate of the erythrocytes (E.S.R.) is an investigation so easy to carry out that it has been used extensively both in the laboratory and at the bedside of the patient without much attention being paid to the variation in results obtainable with different methods, and the pitfalls liable to be encountered in the absence of a standard technique.

Much importance was attached to this investigation by early workers until it was realized that an increased sedimentation rate is only an indication of ill-health comparable to fever or leucocytosis, and therefore not reliable for differential diagnosis.

The E.S.R. is found to be increased in most infections acute or chronic in the presence of tissue destruction, in toxæmia and carcinomatosis, after injections of vaccines and foreign proteins, operations, fractures or irradiation. High values are found in all diseases affecting the osteo-muscular system with the exception of osteoarthritis; it is also raised in pregnancy whether normal or complicated.

The sedimentation rate is found unaffected in appendicitis during the first 24 hours of illness, pertussis and pernicious anaemia if uncomplicated and in slowly progressing degenerative lesions such as arteriosclerosis.

It shows retardation in polycythaemia, congestive cardiac failure and other conditions showing increased corpuscular volume when correction is not made for the raised number of red cells (see p. 288). It has also been found considerably slowed in Niemann-Pick's disease on account of the high level of the blood cholesterol.

The main value of the estimation of the E.S.R. is in differentiating organic from psychotic diseases: in face of a high E.S.R., other than in pregnancy, the clinician must endeavour to find an organic basis for the symptoms. It is also of great value in prognosis and in assessing progress or the effect of treatment, but especially in this case a standard technique must be used throughout the period of observation. Excellent reviews are available on the technical aspects (Ham and Curtis, 1938; Nicholls, 1942) and on the clinical applications (Reichel, 1936) of the E.S.R.

There does not seem yet to be agreement as to the best means of carrying out and recording the rate of sedimentation of the red

cells and many different techniques have been advocated ; results obtained with one method are not comparable with those obtained with the others.

The methods of recording the sedimentation rate of the erythrocytes fall into two categories , the one reports the fall of the red cells within a fixed time (1 minute, 1 hour, etc.—Westergren, Rourke-Ernstene, etc.), the other the time taken by the red cells to reach a fixed mark (18 mm.—Linzenmeier). The various techniques recommended from time to time are all variations of one or other system of recording.

The sedimentation of the red cells is due to the formation of aggregates of erythrocytes, the size of which depends on the activity in the plasma of a factor which has been called by Day (1940) " sedimentin " ; its nature is unknown, but it appears to be related to several of the plasma constituents ; the estimation of the E.S.R. is only an expression of the activity of this factor.

When a column of blood taken with an anti-coagulant is observed for a period of time and the results of the observation plotted on graph paper it will be found that the sedimentation of the red cells occurs in three stages. The first is the time during which the red cells begin to aggregate in clumps and to fall towards the bottom of the tube with increasing speed until the second stage is reached, " the period of constant fall." In this stage the aggregates of red cells have reached their maximum size and their speed of sedimentation remains unaltered up to the time when " packing " intervenes and the third stage begins. During this sedimentation slows down and finally ceases and the blood is eventually divided into clear plasma and red blood corpuscles.

If the more commonly used techniques be employed on the same specimen of blood it will be seen, as shown in Fig. 16, that not only the recorded results, but also the falls as mm. in one hour and the maximal velocities are so different as to make results hardly comparable. This difference is due to the effect of some of the factors which influence the sedimentation rate of the red cells, the most important of which are :

(1) **Size and Position of Tubes :** Once the period of " constant fall " has been reached the rate of sedimentation would remain unchanged if " packing " did not supervene. Therefore to ensure reliable results especially in cases with very high E.S.R. the column of blood should be as high as possible ; a height of 100 mm. as given by the Wintrobe sedimentation tube is sufficient in many cases, but the 200 mm. of the original Westergren apparatus is preferable.

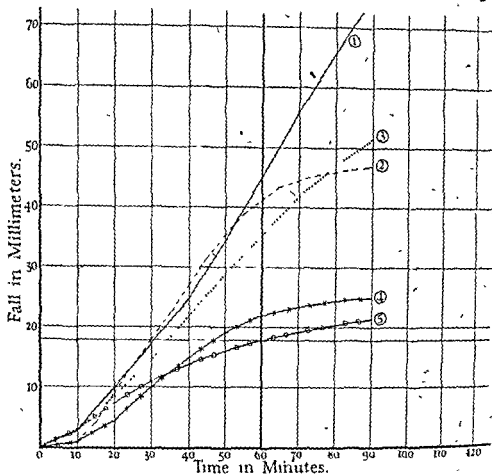


FIG 16 (For description see opposite →)

The internal diameter of the tubes should, to avoid capillary action, be more than 2.5 mm., and the column of blood should be perfectly vertical.

(2) Corpuscular Volume and Size of the Red Cells: In the presence of anaemia each red cell will be surrounded by a relatively increased amount of plasma and therefore also of the "serum" factor. It follows that the E.S.R. of cases with corpuscular volume below 45 per cent. will show higher values than when the same blood is adjusted to a normal proportion of 55 parts of plasma to 45 parts of red cells. Fig. 17 shows the effect of varying the concentration of red cells in the same plasma: the more anaemic is the blood the higher is the E.S.R. expressed either in mm. fall per hour or as fall per minute during the period of constant fall. In cases where the corpuscular volume is higher than normal (polycythaemia, cardiac

FIG 16

Corpuscular volume = 43 per cent.

Technique	Height of column in mm	Anticoagulant	Maximum velocity in mm. per minute	Report
(1) Della Vida	200	Oxalate mixture.	1.05	S R. = 1.05 mm S I. = 2.02
(2) { Rourke- Ernstene Wintrobe	100	Oxalate mixture.	0.90	0.9 mm. per minute.
	100	Oxalate mixture.	0.90	41.5 mm. in 1 hour.
(3) Westergren	200	Sodium citrate 3.8 per cent. 1 part to 4 parts of blood.	0.70	35.5 mm. in 1 hour.
(4) Cutler	50	Sodium citrate 3 per cent. 1 part to 9 parts of blood.	0.55	Diagonal curve
(5) Linzenmeier	50	Sodium citrate 5 per cent. 1 part to 4 parts of blood.	0.45	60 minutes to 18 mm. mark.

failure, etc.) other factors, such as viscosity, enter into play and produce a retardation of the sedimentation rate which is not due only to the raised number of red cells. In these cases it is therefore advisable to adjust the corpuscular volume to 45 per cent. or slightly less before estimating the sedimentation rate. The size of the red cells also has an influence, although small, on the rate of sedimentation and it is preferable to take blood in anticoagulants which will maintain unaltered the size of the cells.

(3) **Anticoagulants:** The anti-coagulant influences to a greater or lesser extent the rate of sedimentation. Citrate solutions slow the E.S.R. to a considerable degree (see Fig. 16); dry potassium oxalate produces a shrinkage of the red cells thus giving an inaccurate reading of the corpuscular volume. Ham and Curtis have shown that only heparin and dry oxalate mixtures as advocated

by Heller and Paul (1934) (commonly known as the Wintrobe salt mixture) give comparable results and at the same time prevent shrinkage of the red cells

(4) Time : The activity of the "sedimentin" shows a progressive though small reduction during the first 4 hours from the taking

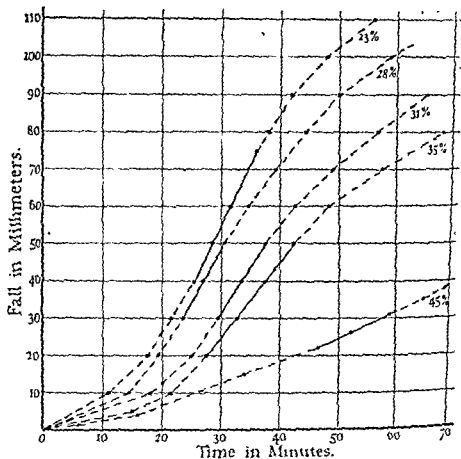


FIG. 17

Corpuscular volume	45 %	= 0.72 mm	per minute	S.I. = 1.86
"	35 %	= 2.00 mm.	" "	S.I. = 1.94
"	31 %	= 2.50 mm.	" "	S.I. = 1.91
"	28 %	= 2.85 mm.	" "	S.I. = 1.85
"	23 %	= 3.33 mm.	" "	S.I. = 1.82

of the sample ; thereafter decrease is rapid until after from 24-48 hours it has almost completely disappeared. This phenomenon is due primarily to the ageing of the red-cells which lose their susceptibility to clumping ; the "sedimentin" activity of the plasma remains unchanged for long periods, and it is therefore possible to

estimate fairly accurately the E.S.R. of specimens of blood older than 4 hours by combining the plasma with freshly drawn washed red cells.

(5) **Temperature:** It has been shown that variations of temperature considerably influence the rate of sedimentation, and it is advisable in laboratories in which there is much seasonal variation of temperature to use an incubator at about 18-20° C.

Technique (Della Vida, 1942)

Blood is drawn from a vein in the antecubital fossa and mixed in a test tube containing dry oxalate mixtures in the proportion of 5 cc. of blood to 10 mg. of oxalates (4 mg. potassium oxalate and 6 mg. ammonium oxalate), taking care to avoid frothing.

A sedimentation tube of the Westergren type is filled to the 200 mm. mark and set up in a vertical position; at the same time the corpuscular volume is ascertained by the haematocrit. When the blood shows signs of sedimenting in the test-tube it is gently but thoroughly mixed before filling the sedimentation pipette. When using the Wintrobe sedimentation tube which gives a blood column of 100 mm. the corpuscular volume may be ascertained in the same tube after the rate of fall has been recorded.

The rate of fall is observed until "packing" begins or until the period of constant fall has been reached, by recording either the height of the plasma column every 5-10 minutes or, as more conveniently in samples showing high E.S.R., the time at every 5 mm. fall. The figures are plotted on graph paper (Fig. 18) and from the straight line expressing the period of constant fall the sedimentation rate (S.R.) is calculated and expressed as mm. fall per minute. In the presence of anaemia the observed figure must be corrected and this can be done either with charts constructed by means of logarithmic curves or more simply by making use of the "sedimentin index" suggested by Day.

The "sedimentin index" (S.I.) is the logarithm of the figure expressing the constant rate of fall over a period of 100 minutes. Correction for anaemia is done by arithmetical proportion taking 45 per cent. as the normal corpuscular volume (C.V.) according to the formula:

$$\text{S.I.} = \log. (\text{S.R.} \times 100) \frac{100 - 45}{100 - \text{C.V. observed}}$$

and the corrected S.R. will be

$$\text{S.R. (corrected)} = \frac{\text{antilog S.I.}}{100}$$

Normal figures for the E.S.R. are from 0.05 mm. or less per minute to 0.25 mm. per minute, giving a sedimentin index of from 0.5 or less to 1.4. Any figure giving a sedimentin index of above

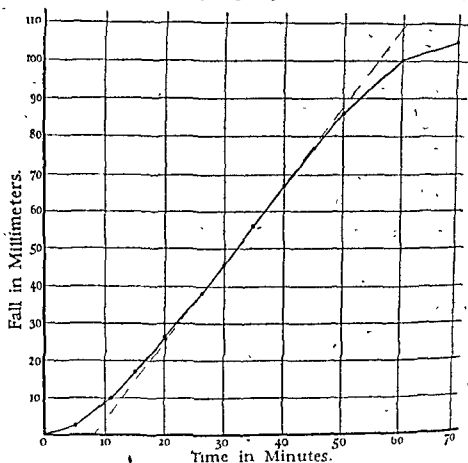


FIG. 18

Corpuscular volume = 36 %.

Observed S.R. = 2.1 mm per minute, S.I. = 2.322.

Correction for anaemia:

$$\text{S.I. (corrected)} = \frac{2.322 \times 55}{64} = 1.995$$

$$\text{S.R. (corrected)} = 0.99 \text{ mm. per minute}$$

1.5 must be regarded with suspicion, although it must be borne in mind that a small infection such as a common cold may produce an increase of the S.I. to a figure of about 1.5.

B. L. DELLA VIDA.

THE SEDIMENTATION RATE

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CHAPTER XXVIII

THE DIAGNOSIS OF PERNICIOUS ANAEMIA AND THE ASSAY OF LIVER EXTRACTS

THE development of pernicious anaemia depends upon a failure in the elaboration of the anti-anaemic factor necessary for the maturation of the erythrocytes. The evidence is that this factor is elaborated in the gastric mucosa and stored in the liver; its exact chemical nature remains unknown. The only means as yet available of determining the presence of the anti-anaemic factor and of assaying the activity of preparations of it is by the response of the blood in a case of pernicious anaemia; conversely the ultimate diagnosis of pernicious anaemia depends upon the response of the blood to the exhibition of the anti-anaemic factor.

Once failure in the elaboration of the anti-anaemic factor resulting in pernicious anaemia is established it persists. The diagnosis of pernicious anaemia carries with it the certainty that the patient must continue treatment with the anti-anaemic factor throughout life. On this account it is desirable that the diagnosis be not made except on conclusive evidence. Further, since treatment with the anti-anaemic factor rapidly destroys the evidence of pernicious anaemia it is essential that the initiation of such treatment be withheld until the diagnosis of pernicious anaemia has been established or disproved with certainty.

DIAGNOSIS

Pernicious anaemia may be defined as a megalocytic anaemia, invariably associated with achlorhydria and responsive to treatment with the anti-anaemic factor. It cannot be certainly diagnosed either on clinical grounds or by any single laboratory procedure. Accurate diagnosis calls for sustained institutional observation.

It is well to open the investigation with the examination of the gastric secretion obtained by means of a fractional test meal, preferably preceded by the injection of histamine (0.01 mg. per kg. body weight). The presence of free HCl even in minimal amounts excludes at once the diagnosis of pernicious anaemia.

The full examination of the blood demands:

- (1) Estimation of the haemoglobin percentage;
- (2) Enumeration of the red cells;

- (3) Enumeration of the white cells ;
- (4) Examination of stained films ;
- (5) Calculation of the colour index ;
- (6) Calculation of the mean diameter of the red cells ;
- (7) Calculation of the mean corpuscular volume of the red cells ;
- (8) Estimation of the serum bilirubin.

Examinations (1) and (2) give essential data ; (3) is of little importance and (4) provides only presumptive evidence. The number of the white cells (3) is usually somewhat diminished. The stained film (4) will show fully chromic red cells, manifesting anisocytosis and poikilocytosis in direct proportion to their diminution in numbers ; the white cells will show a high preponderance of mature polymorphonuclears with four or five nuclear lobes, but in spite of a widespread conviction to the contrary it is impossible to diagnose pernicious anaemia by examination of the stained film only.

The essential characteristics of the blood picture are brought out by examinations (5), (6), (7) and (8).

(5) **THE COLOUR INDEX.** For the reason that the red cells are larger than normal and at the same time saturated with haemoglobin the colour index is always unity or above.

(6) **THE MEAN DIAMETER OF THE RED CELLS (M.C.D.).** This is invariably raised, usually being round about or above $8\ \mu$.

(7) **THE MEAN CORPUSCULAR VOLUME (M.C.V.)** is always raised giving a figure of 100 $\text{c}\mu$ or more. It is obtained by estimating the corpuscular volume (C.V.) with a haematocrit and applying the formula :

$$\text{M.C.V.} = \frac{\text{C.V.} \times 10}{\text{Red cells in millions per c.mm.}}$$

(8) The serum invariably gives a **POSITIVE INDIRECT VAN DEN BERGH REACTION** ; serum bilirubin is always above normal levels but not necessarily grossly so.

The characteristic findings (5), (6) and (7) all depend upon the megalocytosis ; finding (8) depends upon the fact that throughout the active course of the disease there is a continuous haemolysis of the abnormal red cells. Failure to demonstrate any one of these characteristics is strong evidence against a diagnosis of pernicious anaemia. Even in the presence of all of them conclusive diagnosis must depend upon the response to the exhibition of the anti-anaemic factor.

This response is demonstrable first in the bone marrow. In pernicious anaemia this always shows megaloblasts in large numbers. Within twenty-four hours of the parenteral administration of an effective dose of the anti-anaemic factor these will all have disappeared and the myelogram (see p. 246) will present a normal picture. For the reason that it demands two sternal punctures and further that it gives no indication as to the required dosage of the particular preparation of the anti-anaemic factor used this procedure is not recommended for diagnosis.

ASSAY OF THE ANTI-ANAEMIC FACTOR

Commercial preparations of the anti-anaemic factor vary widely in their potency. For this reason it is highly desirable for the purpose of diagnosis and treatment of pernicious anaemia to have always available a preparation of known potency.

The only means of assay of the anti-anaemic factor at present available is by its effect upon the blood in a case of pernicious anaemia. The earliest effect observable in the blood is the "reticulocyte crisis"—a rapid rise, followed by an equally rapid fall in the number of reticulated red cells or reticulocytes. This is followed by a slower rise in the numbers of the red cells.

After parenteral administration of the anti-anaemic factor the reticulocyte crisis occupies from the fourth to the sixth day from injection. After oral administration it is delayed by two or three days.

Efficient dosage with the anti-anaemic factor produces a reticulocyte crisis in inverse ratio to the initial red cell level. The height of the expected rise can be calculated from the formula originally suggested by Minot (1928) and his co-workers and subsequently modified by Riddle (1930, 1940)—

$$R = \frac{0.73 - 0.2 E_0}{0.73 + 0.8 E_0}$$

in which R multiplied by 1000 represents the expected maximum level of the reticulocytes per 1,000 red cells and E_0 the initial number of red cells in millions per c.mm. Optimal dosage with the anti-anaemic factor will usually produce a reticulocyte crisis closely approximating to this formula.

Della Vida (1942) has made it evident that maximal response according to this formula does not necessarily indicate optimal dosage, and conversely that failure to reach the maximum demanded

by the formula may occur with optimal dosage. The ultimate criterion of potency must depend upon the response of the red cells; optimal dosage should bring the red cells to a normal level in from four to five weeks. Experience has shown that the dosage for the first two weeks of treatment may be concentrated in the first day. Optimal treatment produces a rise in their initial level. In the meet Della Vida's modification of Riddle's formula :

$$I = 0.93 - 0.214 E_0$$

in which I represents in millions the average weekly increase of the red cells during the first two weeks of treatment and E_0 the initial number of red cells in millions per c.mm. For therapeutic purposes only those preparations of the anti-anaemic factor for parenteral use can be regarded as satisfactory of which dosage during the first three days of treatment will produce a rise of the red cells satisfying Della Vida's formula by the end of the second week. Certain available commercial preparations will satisfy this requirement in a dose of 1 cc. on the first day; others are very far from doing so.

Whether a preparation produces the expected reticulocyte response or not its worth cannot be regarded as proven until the rise in the red cells has been observed for over two weeks from the commencement of treatment. If this rise fails to continue or if the expected level is not reached at the end of this time an optimal dose of a preparation of known potency must be administered and the reticulocytes and the red cells further observed. The occurrence of a second reticulocyte response indicates that the initial dosage was sub-optimal.

When in making assay of liver extracts failure of response on the part of the blood is encountered the possibility of resistance on the part of the subject must also be borne in mind. Such resistance may be due to active intercurrent disease but will only occur in the presence of morbid conditions so severe as to be obvious. It may be and not infrequently is due to lack of some other substance, as well as the anti-anaemic factor, essential for haemopoiesis. Of such deficiencies the commonest as pointed out by Dyke, Della Vida and Delikat (1942) is vitamin C.

It is a wise precaution to saturate all patients with ascorbic acid before attempting assay of any preparation of the anti-anaemic factor; for this purpose a dose of 100 mg. daily for one week

followed by 50 mg. daily is ample. Thyroxin is also essential for haemopoiesis which will not proceed satisfactorily until hypothyroidism, if present, has been rectified by appropriate treatment.

"Units" of Anti-anaemic Factor

It would be a matter of considerable convenience if the potency of preparations of the anti-anaemic factor could be expressed in terms of units. This is attempted in the U.S. Pharmacopoeia which regards as a unit that daily dose which at the end of fifteen days of treatment will produce an "adequate haemopoietic response." The determination of adequacy is partly based upon the reticulocyte response. Emery and Hurran (1945) have recently suggested that the assay of unitage should be based upon the rise of the red cells as demanded by Della Vida's formula; they recommend that the unit be established as one-fourteenth of the dose of extract which, given at the initiation of treatment will produce a rise of the red cells satisfying this formula at the end of fourteen days of treatment. On this basis a preparation producing such a response in a dose of 1 cc. would be regarded as having a strength of 14 units per cc.; a preparation requiring initial dosage of 10 cc would have a strength of only 1.4 units per cc.

Techniques

Estimation of Mean Diameter of Erythrocytes (M.C.D.):

The ideal method of estimating the M.C.D. is by the construction of a Price-Jones curve of red cell diameters, this is a laborious procedure demanding special apparatus. For clinical purposes "halometry" as originally suggested by Pijper (1919) is satisfactory. The underlying principle is the diffraction of light by evenly dispersed particles, in this case the erythrocytes spread on an ordinary blood film. Various portable halometers of the type devised by Eve are on the market. A simple laboratory halometer may be constructed as suggested by Merlyn Price (1929) by using two sources of light set at a fixed distance apart. On observing these lights through a thin, evenly spread, dry blood film they each appear surrounded by a series of concentric spectral rings. The distance of the components of the two spectra from the centre of the source of light is, as shown by Millar (1926) proportional to the diameter of the diffracting red cells according to the fo

In practice θ is measured by the distance from the lights at which selected components of the two spectra just impinge upon each other. The components to be used are the *inner red bands*.

The source of light is supplied by two frosted electric light bulbs set at a distance from centre to centre of exactly 28 inches (or, if the metric system be used, 58.4 cm). These are covered with a black matt screen having openings for the lights of half an inch (metric, 1 cm) in diameter. The lights are set at eye level on a wall so as to allow observation up to 18 feet (metric, 5 metres) from the lights. The spectral rings seen surrounding the lights on observation through a suitable blood film separate as the observer nears the light and converge as he recedes from it. Readings are made on the inner red haloes by noting the distance from the source of the light at which they just touch one another. The distance in feet divided by two (metric, in metres multiplied by two) gives the mean diameter of the red cells in μ . The observation is best made through a cylinder blackened on the inside, with a slot in which to insert the slide, the film should be turned away from the observer's face to avoid damage by the breath.

The Reticulocyte Count: Solutions required—(A) Brilliant Cresyl Blue and (B) Potassium oxalate, both in 1 per cent solution in normal saline. Keep separately. For use add 5 drops of A to 25 drops of B in a centrifuge tube. The prepared solution must be used within 6 hours. Introduce 8-10 drops of blood, allow to stand for half an hour, centrifuge. Pipette off most of the supernatant solution leaving sufficient for resuspension of the red cells. Resuspend, transfer small drops to slides and make films in the usual way. These may be examined without further staining or after counterstaining with Leishman or some similar haematological stain. Using a square adjustable eye piece count the reticulocytes against the red cells and express as proportion of 1,000 red cells.

S. C. DYKE.

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CHAPTER XXIX

INFECTIOUS MONONUCLEOSIS AND THE DIFFERENTIAL PAUL BUNNELL TEST

ALTHOUGH infectious mononucleosis had been described under the name of "glandular fever" in 1889 (Pfeiffer) and the characteristic blood changes have been well known since 1923 (Tidy and Daniel, Downey and McKinley) it is only since the discovery of the presence of heterophilic antibodies against sheep's red cells in the serum of patients suffering from this disease (Paul and Bunnell 1932) and the introduction of the "differential" Paul Bunnell test (Davidsohn, 1937) that it can be distinguished with certainty from other conditions giving rise to similar clinical and blood pictures. The aetiology of the condition is unknown; it is moderately infectious and may occur either sporadically or in small epidemics; it is more frequent in males and rare after the age of forty; it takes a more acute form in children than in adults; the incubation period is usually from 5 to 10 days but may be much longer.

Clinical Picture

The clinical picture is variable, the most constant feature being generalized glandular enlargement; the glands are discrete, soft and elastic, sometimes very tender. Glands on the left side of the neck are more regularly involved than those of the right side.

Tidy (1934) has attempted to divide infectious mononucleosis according to the presenting syndrome into three types, the glandular, the anginous and the febrile.

The glandular type has a sudden onset with fever, headache and malaise and sometimes a rigor. Petechial haemorrhages and signs of meningeal irritation may occur. Between the fourth and tenth day a pinkish maculo-papular rash may appear mainly on the front of the trunk. Several crops of rash may precede glandular enlargement. The spleen is usually enlarged and soft. Jaundice is not unusual. The involvement of mesenteric and thoracic glands may give rise to abdominal or chest symptoms.

In the anginous type sore throat is the presenting symptom and may be very severe.¹ It may take the form of follicular tonsillitis,

¹ SMITH, K. S. & SHAW, T. H., in *Brit. med. J.* 1. (1945) 581, describe the very striking curative effect of a single moderate to full dose of one of the intravenous arsenicals in severe forms of the anginous type --(Ed)

of hyperaemia of the fauces or be of membranous type simulating diphtheria.

The febrile type gives a clinical picture similar to that of typhoid fever.

The only complication reported is haemorrhagic nephritis.

Blood Picture

In the early stages of the disease blood changes may not be characteristic. There may be moderate leucocytosis with increase of polymorphonuclears. Sometimes there is leucopenia throughout the disease. The characteristic blood picture when established is leucocytosis of up to 40,000 white cells per c.mm. with increase in the number of mononuclear cells. These consist of normal lymphocytes, normal monocytes and the so-called "glandular fever cells." These latter are very variable in size and structure and three main types can be recognized. In the first the nucleus is indented or lobulated with coarse and irregular chromatin similar to that of the plasma cell; there is marked contrast between the deeply basophilic spongioplasm and the pale hyaloplasm, which is arranged mainly around the nucleus. In the second type the nucleus is less irregular in shape, the spongioplasm is less dense and less basophilic and is distributed at the periphery of the cell in large cloudy masses or is arranged radially from the nucleus, it may contain a few vacuoles filled with badly defined hyaloplasm. The third type, which is rather rare has a more immature nuclear structure with diffuse chromatin and sometimes with nucleoli. These abnormal cells may persist in the circulation for several months after the subsidence of symptoms.

At the height of the disease the Wassermann reaction may become positive.

The bone marrow may show an increase in the number of mononuclear cells due to the presence of cells similar to those in the bloodstream (Freeman, 1936), but whether these cells are actually formed within the marrow itself or are from the blood mixed with bone marrow during aspiration remains uncertain (see p. 254).

The Paul Bunnell Test

The conclusive diagnosis of Infectious Mononucleosis depends upon the demonstration in the serum of heterophilic antibodies to sheep's red cells; this goes by the name of the Paul-Bunnell test. These heterophilic antibodies may be present from the fourth day

of the disease but often do not appear until the glands are enlarged. They are not of the Forssman type since unlike these, they are not absorbed by the tissues of the guinea-pig; on this fact depends the differential test.

Antibodies against sheep's red cells may occur in the serum of normal people but usually to a low titre only. After the injection of horse serum true Forssman antibodies against sheep's red cells may appear in the serum to a very high titre. For this reason the simple Paul Bunnell test does not suffice to differentiate the heterophilic agglutinins of infectious mononucleosis from those induced by injection of therapeutic sera or from those occurring in normal serum. Davidsohn and Walker (1935) noticed that while the antibodies appearing after a dose of horse serum can be completely removed from the blood by absorption with guinea-pig's kidneys, those specific to infectious mononucleosis are only very slightly affected. If ox red cells are used for absorption both these agglutinins are removed, but those occurring in normal serum remain unaffected. Therefore complete removal of agglutinins against sheep's red cells by absorption with ox red cells and failure to remove them by absorption with guinea-pig kidney is characteristic of infectious mononucleosis.

Why the typical heterophilic antibodies should appear in the serum in infectious mononucleosis is quite unknown and it is therefore impossible to state whether or not their elaboration represents an essential feature of the disorder, but in the present state of knowledge it is probably wise to confine this diagnosis to cases showing, together with the typical clinical and blood pictures, the presence in the serum of the characteristic heterophilic agglutinins against sheep's red cells, susceptible to absorption by ox red cells but not by guinea-pig kidney.

Technique of the Differential Paul Bunnell Test

Preparation of Boiled Ox Red Cells: Wash the red cells three times with normal saline. Resuspend the packed cells in four volumes of normal saline, boil for 1 hour in a water-bath. Cool and make up for evaporation with distilled water. Add phenol up to a final concentration of 0.5 per cent.; keep in the refrigerator. Shake before use.

Preparation of Boiled Guinea-pig Kidney: Remove perirenal fat, capsule and pelvis. Keep kidneys frozen in refrigerator. For use thaw and wash repeatedly in normal saline until the washings are free from blood. Mash into a fine pulp; add four volumes of normal saline; boil for 1 hour in water-bath. Cool and make up for evaporation with

distilled water. Add phenol to a final concentration of 0.5 per cent.; keep in the refrigerator and shake before use

Both emulsions will keep on ice for many months

The Test: Inactivate the patient's serum by heating for 30 minutes at 56° C.

Into three centrifuge tubes introduce 0.1 cc. of the patient's serum. To the first (control) add 0.4 cc. of normal saline, to the second 0.4 cc. of the guinea-pig kidney emulsion and to the third 0.4 cc. of the boiled ox red cells. Leave at room temperature for 1 hour, shaking the second and third tubes every 15 minutes; centrifuge these two tubes and retain absorbed supernatant fluids

For each fluid set up a row of six small (approx. 6×1 cm.) test-tubes, each containing 0.25 cc normal saline. In the first tube in the first row place 0.25 cc of the control diluted serum; in the first tube in the second row place 0.25 cc. of the serum absorbed with the guinea-pig kidney and in the first tube in the third row, 0.25 cc. of the serum absorbed by the ox red cells. Mix well and make serial dilutions along each row of tubes by transferring 0.25 cc. from the first tube of each row to the second, 0.25 cc from the second to the third and so on to the sixth tube in each series, discard the 0.25 cc from the last tube in each row. To each tube add 0.1 cc of a 2 per cent. suspension of washed sheep's red cells. Final dilutions are 1:14, 1:28, 1:56, 1:112, 1:224 and 1:448. Leave at room temperature for 2 hours. Shake, examine for presence of agglutination both by naked eye and under the low power of the microscope.

ALŽBETÁ DELIKATOVÁ.

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CHAPTER XXX

THE ANALYSIS OF THE SEMEN

UNTIL recently it was largely taken for granted that in barren marriages it was the female partner alone who was at fault. Little attention was paid to possible "impotentia generandi" in the presence of "potentia coeundi." Investigations of the last score of years have greatly changed our outlook in this respect. It has now become customary to examine both partners in any case of impaired fertility. So far as the male partner is concerned the laboratory investigations consist in the analysis of the semen.

COLLECTION OF SPECIMEN

Since the purpose of the examination lies in the assessment of the viability of the spermatozoa due care must be taken to protect the specimen after it has been voided from any harmful influence. For this reason attention must be paid (a) to the conditions under which the specimen is obtained, (b) to the container into which it is voided and brought to the examiner and (c) to the conditions under which the specimen is transported.

Samples for examination may be collected in three different ways: (1) by masturbation; (2) by intercourse with a rubber sheath; (3) by coitus interruptus.

The patient is told to refrain from intercourse for five days preceding the collection of the specimen. If the patient raises no objections it is advantageous to choose the method of masturbation within the examiner's premises as no special arrangements need be made, no transport is necessary and the specimen can be examined almost at once. The sample should be collected directly into a wide mouthed dry glass jar.

If either intercourse with a rubber sheath or coitus interruptus is chosen special care must be taken as to the condition of the rubber sheath or the glass jar into which the specimen is voided. It is a common practice to impregnate the rubber used for condoms with chemicals which prove deleterious to the life and motility of the spermatozoa; it is therefore advisable to recommend frequent rinsing in running water and subsequent thorough drying of the sheath before use. In the case of a glass jar it is preferable to use an old glass jar which has been frequently rinsed out in the past

rather than new glass, as the latter sometimes liberates substances injurious to sperm motility.

The specimen should be examined as soon as possible after collection, preferably within an hour. As to transport, it should always be kept in mind that spermatozoa can stand low temperatures for quite a long time without any damage, whereas their viability is likely to suffer by exposure to higher temperatures. The higher the temperature the sooner the deleterious effect will become noticeable. If a given sample is divided into two parts and the one kept for several hours at body temperature while the second is put into the ice-box, the spermatozoa of the latter will show the better motility after having been warmed up again. If a specimen has to be transported over long distances special care should be taken to keep it as cool as possible. It has been found convenient to immerse the water-tight container into a thermos flask filled with cold water.

EXAMINATION OF SPECIMEN

The semen consists of two fractions, the cellular elements originating from the testicles, and the liquid medium which is produced by the accessory glands and in which the cells are suspended when ejaculation takes place. Routine investigation consists of macroscopic examination of the semen and microscopic study of the cellular elements.

I. Macroscopical Examination of the Semen

On receipt of the specimen the following characteristics should be noted before starting more detailed investigations: (a) the volume; (b) the physical appearance, *i.e.* colour, smell, absence or presence of liquefaction and viscosity.

Volume of the Specimen: This is determined by drawing the fluid up into a graduated pipette; the semen should then be returned into the original container. The average normal volume of any ejaculate varies between 2 and 5 cc. The amount of the ejaculate is no criterion of its fertility unless it is less than 1 cc. and is associated with other abnormal findings.

Physical Appearance: Immediately after ejaculation the normal specimen is a thick whitish viscid fluid which liquefies after standing at room temperature for 15-30 minutes.

Variations in colour occur; specimens collected after prolonged sexual abstinence have a slightly yellow tinge. Pyospermia and

haemospermia can occasionally reach such degrees that they will be noticeable on macroscopic inspection.

The transient initial viscosity is due to the presence of certain proteids in the seminal fluid. The subsequent liquefaction is the result of the activities of enzymes and bears no relation to the number and viability of the spermatozoa present. Cases have been reported which showed a high initial viscosity remaining unchanged without liquefaction; if, however, a few drops from a normally liquefying specimen were added liquefaction of the first sample could be induced; the liquefying property of the second specimen could be destroyed by heat. This is a further indication of the enzymatic character of this process.

In recent years the presence of hyaluronidase in seminal fluid has been demonstrated. Whether or not the liquefaction of semen and the dissolution of the cervical plug is due to this substance has yet to be proved.

The biological significance of abnormally high viscosity and failure to liquefy has been much disputed. It has been claimed that increased viscosity reduces the chances of spermatozoa reaching the cervical canal. It is a fact that sperm motility is greatly influenced by the viscosity of the medium in which they are suspended.

Increased viscosity and failure to liquefy are abnormalities in the seminal fluid due to disturbed function of the accessory glands probably primarily caused by some endocrine dysfunction. Decreased viscosity also occurs. This condition, liquefaction taking place immediately after ejaculation, is very suggestive of oligo- or azoospermia.

Determination of the Hydrogen Ion Concentration: The average pH of a freshly passed specimen varies between 7.2 and 8.5. Fertile and sterile specimens show no significant difference in the hydrogen ion concentration and it is not necessary to include pH determinations in routine semen analysis.

In a few cases it may be important to determine the pH; this may be done either by the electrometric or the indicator method. The only equipment necessary for the indicator method are buffer solutions and indicators covering the range from about pH 6.5 to pH 9.0. As a comparatively small amount of material is available for examination it is necessary to use a micromethod, such as the spot method quoted by Meaker (1934). It is convenient to have a white porcelain or glass plate with several depressions similar to the concavities of "hollow slides." The test is carried out by using the buffer solutions of Clark *et al.* (1916) (see Tables 1 and 2).

TABLE 1

To 50 cc of M/5 acid potassium phosphate add the indicated number of cc. of N/5 NaOH and dilute to 200 cc.

pH	NaOH	pH	NaOH	pH	NaOH
6.4	12.60	7.0	29.63	7.6	42.80
6.6	17.80	7.2	35.00	7.8	45.20
6.8	23.65	7.4	39.50	8.0	46.80

Indicators. Bromcresol purple, bromthymol blue and phenol red..

TABLE 2

To 50 cc of M/5 boric acid in M/5 KCl add the indicated number of cc of N/5 NaOH and dilute to 200 cc.

pH	NaOH	pH	NaOH	pH	NaOH
7.8	2.61	8.4	8.50	9.0	21.30
8.0	3.97	8.6	12.00	9.2	26.70
8.2	5.90	8.8	16.30	9.4	32.00

Indicators : Cresol red and thymol blue.

A drop of semen is placed in the central hollow of the white plate and one drop of one of the buffering solutions is put into each of the surrounding hollows. A separate plate should be prepared for each indicator. An equal amount of indicator is added to each buffering solution and to the drop of semen and the colours compared. In most cases it will be sufficient to use one plate with cresol red as indicator.

Determination of the Buffering Capacity : There has been much controversy as to the importance of the buffering capacity in the survival time of the spermatozoa. It is now generally agreed that under normal conditions the buffering capacity is high enough to ensure the survival and motility of the spermatozoa in the acid medium of the vagina. However if the volume of the ejaculate is excessively small (less than 2 cc.) a low buffering capacity may not suffice to neutralize the acid reaction within the vagina; this is especially likely to happen when the ejaculate is deposited in the posterior fornix. It may therefore be useful and even necessary to assess the buffering capacity in routine analysis.

This is done by diluting 0.5-1 cc. of semen with double glass-distilled water and assessing the amount of N/100 HCl necessary to

adjust the pH to 7.1. In some laboratories it is also customary to assess the amount of N/100 HCl needed to turn the pH to the acid side (pH 6.7). In our experience the mean value of the buffering capacity has been found to be 3.1 cc. of N/100 HCl per 1 cc. of semen when the pH of 7.1 is taken as endpoint, with extreme values of 2.2-4.1 cc.

Chemical Constituents: The chemistry of the seminal fluid has been studied by various workers; Roberts *et al.* (1939) have collected the relevant data in Table 6 (p. 43) of their book.

Fertile and infertile specimens show the same range of variation in chemical constituents. It has, however, been claimed that excessive concentrations of phosphate are a fairly common finding in certain types of lowered fertility and sterility. A high concentration of calcium salts and a raised glucose concentration are also characteristic of some cases of lowered fertility. On the other hand there are many cases of definitely impaired fertility which show a perfectly normal chemical composition of the seminal fluid.

Convertible Carbohydrate: Apart from glucose semen contains another substance convertible into glucose by diastase; the identity of it is unknown and it has been called "convertible carbohydrate." The name of *amylospermia* has been given to a condition in which the amount of convertible carbohydrate is greatly increased. It is frequently met with in impaired fertility and the existence of this condition is revealed by the following test:

(1) 0.3-0.5 cc. of seminal fluid is diluted with the same amount of distilled water and the glucose contents determined by one of the usual micromethods.

(2) The same amount of seminal fluid is mixed with an equal volume of 1 per cent. diastase solution and incubated at 37° C. in a stoppered test-tube for two hours; the glucose content is then determined.

(3) The difference between the glucose content of sample 2 and of sample 1 indicates the concentration of convertible carbohydrate.

The normal values for glucose in semen are 200-300 mg. per cent and for convertible carbohydrate 50-70 mg. per cent. In amylospermia there is a significant rise in the concentration of glucose after the addition of diastase (50 per cent. or more) and figures up to 600 mg. per cent. have been recorded. Amylospermia indicates a disturbance of the function of the accessory glands and as such is proof of a disturbance of genital apparatus.

II. Microscopical Examination of the Semen

The cellular elements of a normal seminal specimen consist mainly of spermatozoa; other elements may be found and are regarded as pathological if they occur in appreciable numbers. Such elements are:

(a) Red and white corpuscles indicating inflammation and congestion if present in considerable numbers, and especially if clumped together.

(b) Epithelial cells from the male genital system (tubuli recti, vas deferens, prostate, urethra and bladder), of columnar, squamous or transitional type. They occur singly in normal seminal fluids, but when found in appreciable numbers or in sheets indicate the presence of an inflammatory process. Corpora amylacea of the prostate are a common finding in old age and in conditions of exhaustion. These are laminated round bodies staining with iodine; they are usually $10-30\mu$ in diameter but may be up to 1 mm. and then visible to the naked eye.

(c) Abnormal or immature cells from the testicles themselves. Abnormal spermatozoa will be dealt with later (see p. 312); spermatids are occasionally seen in normal ejaculates, but their presence in any number suggests gross abnormalities. Spermatids are small spherical cells with little cytoplasm and a large round nucleus showing irregular karyosomes. Mononuclear and multinuclear cells are a common finding. Giant cells and cells resembling spermatogonia also occur fairly frequently in abnormal semen.

A preliminary preparation of a drop of semen under a thin cover-glass should be examined under low power magnification to find out if spermatozoa are present at all, if they show motility and finally if there are any gross abnormalities.

The spermatozoa are by far the most important and most interesting constituents of the semen. The final opinion upon a given specimen is based on the number, viability as judged by motility, and morphological appearance of its spermatozoa.

Counting of Spermatozoa: The following simple method is satisfactory: semen is drawn up to the 0.5 mark of a white blood cell pipette and diluted with 1 per cent. formol saline up to the 11 mark. The pipette is well shaken and a blood counting chamber filled with the mixture. The count is then made as for red cells and the result expressed as sperms per cc.

Pollak *et al.* (1939) recommend carbol fuchsin in physiological saline as diluting fluid.

If no spermatozoa are detected after repeated examinations of preliminary preparations, the ejaculate should be thoroughly centrifuged; in extreme oligospermia it is necessary to concentrate the material. In those cases in which no sperms can be detected in the sediment it is advisable to examine coloured smears, because occasionally spermatozoa surrounded by mucus cannot be distinguished in a native preparation. Only when all these attempts give negative results is one justified in speaking of azoospermia.

In normal fertile specimens there are 100-150 millions spermatozoa per cc. Opinion differs greatly as to the number of spermatozoa necessary to guarantee fertility. Some authors hold that a sample with less than 60 millions sperms per cc. is of definitely lowered fertility, while others believe that, everything else being normal, counts as low as 10 millions per cc. are sufficient to ensure fertilization.

Sperm Motility: The assessment of the percentage of motile spermatozoa in any given specimen is subject to many causes of error. The spermatozoa are motionless in the seminiferous tubules and acquire their motility only after being suspended in the seminal fluid derived from the accessory glands.

The degree of motility is dependent both upon temperature and upon the physical and chemical properties of the medium in which the spermatozoa are suspended. On account of the effect of osmotic pressure hyper- and hypotonic solutions must be avoided as diluents for semen.

Motility in itself is by no means identical with the fertilizing power of the spermatozoa. Locomotion of the sperm is produced by the whip-like action of the tailpiece, whereas the fertilizing power is a function of the head. It is therefore conceivable that motionless spermatozoa could fertilise an ovum provided they reach it, e.g. by artificial insemination. Under normal circumstances fertilization cannot be induced by motionless spermatozoa.

The number of motile spermatozoa can be determined either by means of the Ehrlich ocular screen which divides the field of vision into four quadrants, or more conveniently by the method suggested by Roberts *et al.* (1939). This consists in adjusting into the eyepiece a disk of black paper with a narrow sector cut out from it; this narrows down the field of vision and thus facilitates the count. Motile spermatozoa are counted first as quickly as possible, and non-motile ones afterwards. If the motility is very low, below 20 per cent., the count should be repeated after heating the slide to body temperature.

Recently the method of Mrs. Clare Harvey of Exeter has proved the best for assessing motility and viability :—Dilute the semen according to density, 1 in 10 or 1 in 20 with Walton's buffered glucose solution (Na_2HPO_4 2.04 g, KH_2PO_4 0.08 g, glucose 3.2 g, water to 100 cc.). Keep the mixture at 37°C . and test for motility after one, three and five hours respectively. Place drops of the mixture in two warm counting chambers; expose one to 2% osmic acid vapour. Determine the density by counting the killed spermatozoa in the osmicated chamber. Count the non-motile sperms in the untreated slide and by difference estimate the total count per 1 cc of motile forms. Viability is regarded as satisfactory if no appreciable drop of motility occurs within the five hours of observation.

When no motile spermatozoa are found in the preliminary preparation, motility can sometimes be elicited by various methods. Pollak *et al.* (1939) suggest diluting one part of the thoroughly mixed ejaculate with about ten parts of isotonic magnesium salt solution. (Any Mg salt such as MgSO_4 , MgCl_2 or $\text{Mg}(\text{OH})_2$ may be used) The mixing is performed by drawing up the mixture into a pipette and blowing it out again and finally the cavity of a hollow slide is filled up to the brim. The preparation should be examined for at least 10 minutes under the microscope. The use of a slide heated to 38° – 40°C . is helpful in examining specimens with reduced motility.

Morphological Examination : A very important criterion in the evaluation of a given specimen is the relative amount of abnormal forms. The pertinent data are obtained by examining stained smears. The classification in a differential count is as follows :

- (A) Normal adult spermatozoa.
- (B) Abnormal spermatozoa :
 - (a) immature forms,
 - (b) degenerative forms,
 - (c) teratological forms.

(A) NORMAL ADULT SPERMATOZOA consist of four distinguishable parts, namely the head, the neck, the middle piece or body and the tail.

(1) *The head* is about $4\text{--}5\ \mu$ long, is oval or pyriform in shape and exhibits a clear or weakly staining anterior half and a darkly staining chromatin mass in the posterior half. It represents the nucleus of the cell covered by a thin sheet.

(2) *The neck* is the minute part of the spermatozoon which

prepared with 3 parts of a 5 per cent. solution of formalin and 1 part of saturated solution of gentian violet in alcohol, will remain stable for years.

Mönch's Fuchsin-Eosin Method (Meaker, 1934) : The stain is prepared from a stock solution consisting of 50 parts of carbol fuchsin, 25 parts of 95 per cent. alcohol and 25 parts of a saturated solution of eosin in alcohol. 5-10 cc of the stock solution are poured into a staining cup and acid fuchsin (in 2 per cent. acetic acid) is added drop by drop until a fine membrane is formed on the surface ; after filtering the solution is ready for use.

(1) Fix the films by heat and immerse for several minutes in a 0.5 per cent solution of T-chloramine to remove the mucus.

(2) Wash in distilled water and then in 95 per cent alcohol.

(3) Stain with the filtered fuchsin-eosin solution for 2-5 minutes. Wash in tap water.

(4) Counterstain with Löffler's methylene blue for a few seconds.

Some authors (Roberts *et al.*, 1939) recommend that the smears should be made not from the original sample but from the sediment after centrifuging. The semen sample is well stirred and 0.2 cc. drawn up in a pipette and diluted 10-50 times with chloramine-Ringer solution (1 per cent T-chloramine in Ringer solution). The mixture is vigorously shaken and centrifuged at 2,500 revolutions per minute ; the supernatant fluid is then examined for the presence of spermatozoa and if a considerable number is still present centrifuging is repeated. When most of the sperms have collected at the bottom the supernatant fluid is decanted, another 5 cc of the chloramine-Ringer solution is added and the process of centrifuging and decanting repeated. The deposit is thoroughly stirred and thin films prepared.

EVALUATION OF THE SEMEN ANALYSIS

It must be born in mind that it is impossible for the clinical pathologist who examines a single specimen of semen to make a diagnosis as to the fertility of the patient who produced the specimen. All that can be given is an opinion as to the fertility of the sample in question. An unfavourable result of the examination of a single specimen does not necessarily imply a diagnosis of permanently impaired fertility in the patient.

Neither the number, morphology and motility of the spermatozoa nor the physical and chemical properties of the seminal fluid constitute in themselves decisive criteria of the fertility of a given semen sample, the most important factor on which the diagnosis rests is the uniformity, or alternatively, the variation in the sperm population.

The following criteria have been found useful in evaluating the relation between abnormal sperm population and fertility.

(1) Azoospermia and extreme oligospermia exclude fertility.

(2) Reduction in density does not impair fertility provided the spermatozoa present are normal; the probability of fertilization, however, increases with the density.

(3) Motility is not identical with fertilizing power. True necrozoospermia excludes fertility, but this is a rare condition and should not be diagnosed unless a fresh masturbation specimen has been examined and various resuscitation attempts have been made without success (warming of slides, addition of isotonic magnesium salts, addition of vaginal or cervical secretions, etc.).

(4) Normal viability is not necessarily a proof of fertilizing power.

(5) The differential count of the spermatozoa :

(a) the percentage of abnormal head forms is now regarded as a reliable criterion of the fertilizing power; more than 20 per cent. of abnormal head forms is regarded as proof of impaired fertility.

(b) the diagnostic importance of abnormal middle pieces is negligible compared with the conclusions drawn from the presence of a large number of abnormal head forms.

(c) Immature forms in high numbers and the presence of testicular epithelial cells are an indication of impaired fertility.

(d) A specimen not showing isozoospermia should not be regarded as fully fertile.

Summary

As a routine examination the following procedure is suggested:

(1) Description of the gross appearances of the specimen: colour, smell, presence or absence of liquefaction, volume and viscosity.

(2) Examination of a native preparation at room temperature (20° C.) and if necessary at 38° C

(3) Cell count and determination of density.

(4) Assessment of motility; to be repeated after 6, 12, 18 and 24 hours.

(5) Preparation of stained smears and examination of the morphology of the sperm population and occurrence of abnormal forms.

(6) Determination of the pH and of the buffering capacity.

(7) Examination for amyloospermia.

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CHAPTER XXXI

CARCINOMA CELLS IN SPUTUM AND PLEURAL FLUID

THE identification of detached carcinoma cells depends on the principle that they are, in Ludford's (1942) words, "not simply cells that have acquired rapidity of growth, they are specifically altered cells."

No certain differentiating technique has yet been devised for their identification in sputum, but it can only be a matter of time before some fairly simple method makes diagnosis easier. Alterations in cancer cells of such elements as mitochondria, Golgi apparatus and chromosomes have already been amply described and the most promising research would seem to be the exploitation of methods by which the cells can be seen in the vital state, stained or unstained, or by dark ground illumination. These are routine techniques for cancer cytologists and the last named has already been advocated by Gloyne (1937) for sputum. For the time being a fresh wet preparation of sputum stained with methylene blue presents many advantages over other techniques or stains.

Technique: Place the sputum in a Petri dish on a dark background and pick out on a glass slide a small portion with a pair of forceps; a magnifying glass is useful for avoiding extraneous material. One or more slides may be prepared.

The stain, which should be filtered before use, is made of:

Methylene blue	0.5 g.
Distilled water	80 cc.
Glycerine	20 cc.

The glycerine helps the stain to remain in the cells in case of delay in examination and is not essential. Cancer cells do not decompose so quickly as is generally supposed and though a fresh specimen is preferable it is quite possible to recognise them after several days.

Place a drop of stain beside the particle of sputum and gradually introduce it with the aid of a pointed instrument (a blade of the forceps is handy). Mix very thoroughly and gradually until the whole is dark blue and quite sticky; this may take 3 or 4 minutes. Drop coverslip lightly upon the preparation and leave for 10 minutes for the stain to soak in. The material should be so placed that it comes to the centre of the coverslip and spreads evenly towards the edges. When there is sufficient, divide into two parts and use two coverslips. After 10 minutes press the coverslip gently down to thin out the film.

should be uniformly blue though the mucus never takes up the stain quite so deeply as the cells. Examine first with the low power objective to see the lay-out of the constituents and to locate any minute clumps of cells. The high power is required for the identification of the cells but there is no need for the oil immersion objective.

The wet preparation is not permanent and fades after 24 hours. A little glycerinated stain mixed with the sputum in bulk preserves it so that films can be made the next day without further treatment.

For greater permanence a fixed film is necessary. A common procedure is the adaptation of Dudgeon *et al.* (1935) quick diagnostic technique for tumour scrapings.

Make 5 thin films on slides and immerse in a bath of Schaudinn's fluid for 10 minutes

Wash in alcohol with a trace of iodine for 2 minutes

Wash in distilled water.

Stain with Mayer's haemalum for 1½–2 minutes.

Blue with tap water.

Counterstain with eosin for 2 minutes

Dehydrate, clear and mount in Canada balsam

A simpler method which seems to give an equally good picture is to spread the sputum on a slide and let it dry completely in the air. Stain with haematoxylin-eosin as for fixed films. The albuminous nature of sputum renders any other fixation unnecessary.

In a wet film the cells are seen in the round and suffer none of the changes due to fixation. Malignant cells look all the more malignant and their distortion and degeneration is sometimes so flamboyant that they can be recognised even when lying singly. Vacuolation, fatty change and phagocytosis are easily seen and support a clinical diagnosis of carcinoma. Enlargement of the nucleolus is also more obvious in unfixed material.

Red blood cells appear brownish in colour, sometimes crenated. Frankly blood stained sputum is uncommon in carcinoma but a few red cells which would otherwise be missed are often seen in wet films. Plaques of cells have ill-defined divisions and tend to be partially detached; clusters are formless with cells lying on top of one another. Since carcinoma is very liable to necrosis the sputum often contains pale blue fragments of cells with degenerate or absent nuclei; they are found in groups but lie separately, and though not pathognomonic of malignant disease of the lung, they are suggestive of it.

The main distinguishing features of individual cells are:

(1) **Variation in Size:** Some malignant cells are very large, whereas ordinary sputum contains cells which are fairly uniform

and have a large irregular nucleus, usually eccentric, with a large nucleolus. Multiple nuclei may be seen but it must be remembered that endothelial cells sometimes have double nuclei. Mitotic figures, especially if aberrant in form, are very significant and appear more frequently than in sputum.

There is a tendency to fatty change but this is not an infallible sign as macrophages associated with resolving pulmonary inflammation may be full of fat droplets or lipoid material. The special stain for neutral fat is sometimes inconclusive and Gloyne (1937) suggests that "lipoid" would be the most convenient term for all these refractile droplets in degenerate cells.

Vacuolation is not of diagnostic importance as it is frequently seen both in endothelial and macrophage cells undergoing degeneration; nor is phagocytosis, though "rampant" ingestion of smaller cells is very suggestive of malignancy (Dudgeon *et al.*, 1935).

Non-malignant macrophages are usually oval in shape, with granular protoplasm, a central well-formed nucleus and never attached to one another. They usually contain pigment particles such as are never seen in cancer cells.

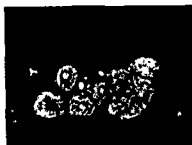
Pleural endothelial (mesothelial) cells must be differentiated from carcinoma cells. They are best examined in the Leishman film where they appear dark blue and several may be fitted together in a pavement pattern quite unlike the loose attachment of carcinoma clusters. The nucleus is large with a clear cut outline and a pale nucleolus; sometimes nuclei are double but this need not give rise to difficulty; they are conventional in arrangement and look like twins. These pleural cells look like their pictures in text-books, whereas illustrations of malignant cells are never satisfactory on account of variations in the type and vitality of the detached elements.

Cells or clusters that suggest malignancy but appear unusual, may be derived from metastases on the pleura from remote organs; this is especially the case when glandular acini are found.

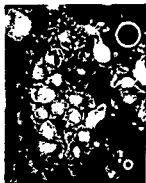
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1



2



3



4



5



6



7

1. Cluster of malignant cells. Poor cell divisions; very large nuclei and nucleoli. $\times 320$
2. Cluster of malignant cells. Loosely attached cells, variable in size and shape, with large irregular hyperchromatic nuclei; one normal squamous cell amongst them. $\times 255$.
3. Cluster of malignant cells. Columnar type, overlapping each other; long, distorted tails; one cell has lost its nucleus. A polymorphonuclear cell in the lower part of the field. $\times 255$
4. Separate malignant cells. Great variation in shape; some columnar cells with long distorted tails. Most of the cells are degenerate and in some the nucleus is absent. $\times 255$
5. Single very long cell with lipid granules and vacuole. Pus cells and one monocyte near by. $\times 255$.
6. Single large cell with very large (? swollen) nucleus and inclusion bodies. $\times 320$.
7. Single large cell, from pleural fluid. Refractile droplets in cytoplasm and large irregular hyperchromatic nucleus. $\times 320$.

SECTION IV
HISTOLOGY

FOREWORD

DIAGNOSTIC histology is the oldest branch of clinical pathology for in 1843, when bacteriology did not exist, clinical biochemistry was still in the realms of uroscopy and haematology unexplored. Julius Vogel published his *Icones Histologicae* setting out the microscopical features by which various disease processes could be recognized and in 1847 Küss was using aspiration biopsy for tumour diagnosis. But seniority is not necessarily an asset in science and many hospital laboratories are still content, so far as histology is concerned, with the technical methods of the end of the last century although eager to encompass the newest advances in the other three branches of clinical pathology. The reasons for this are various, but the principal one is that the contemplative attitude of mind which is the chief attribute of a good histologist is not commonly a characteristic of a successful general clinical pathologist; on the other hand, morbid histologists have been unwilling to regard themselves as clinical pathologists, preferring to stay in the peace of their laboratory and describe structural changes, rather than to go into the wards, operating theatres and follow-up clinics to study the natural history of the disease processes from which the tissues have been derived, blending these observational studies with the modern outlook in biology, and so build up a concept of the functional morphology of disease. But views are changing and most of the essays in this section illustrate the first stage in the renaissance of morbid histology—the integration between the histologist and the clinician in the illumination of obscure corners, whether of diagnosis or prognosis, of disease. It has not been possible, except in an indirect way, to discuss the other and more fundamental aspect of this renaissance—the concept of spatial biochemistry—for as yet the discoveries in this field have little direct bearing on the craft of medicine, but it may well be that the value of histochemistry in clinical pathology can be displayed in the near future.

The concluding essay provides a review of modern clinical histological technique by one who knows and studies this art for its own sake as well as for the assistance it can give in the practical applications of histology and it may seem somewhat anomalous that in the other essays, different methods are given for achieving a similar object; but skilled craftsmen are naturally individualists

and the special method described may be peculiarly suitable for the tissue to be studied. Nonetheless, though one would deplore the introduction of a uniformity in technique for the sake of a tidy standardization with a consequential lowered efficiency, yet it may be that one specialist can learn from another and so simplify and improve the general canon of histological technique.

A. H. T. ROBB-SMITH.

CHAPTER XXXII

ASPIRATION BIOPSY

THE diagnosis of the true nature of a tumour (particularly of a neoplasm) must finally rest upon histological evidence. With the increasing use of radiotherapy it often happens that patients are treated without a definite diagnosis because of the major operation involved in obtaining histological evidence. Yet the clinical and radiological findings, on which the diagnosis must otherwise rest, may be misleading and the treatment given may as a consequence be unsuitable. Moreover, unless the clinical diagnosis is supported in all cases by histological evidence, some doubt as to the correctness of the diagnosis cannot altogether be discounted when the value of treatment comes to be assessed later, particularly five-year cures by radiotherapy.

The necessity for histological proof of malignancy is revealed in a review of the subject by Khanolkar and Sharpure (1944) where it is shown that statistics drawn from various surgical clinics revealed that subsequent histological examination showed an incorrect diagnosis in from 10 per cent. to 57 per cent. of cases.

Aspiration of material for microscopical examination in specific instances is an old procedure, but the elaboration of its uses in the rapid diagnosis of malignant neoplasms is due principally to Martin

Sayago (1942), Iversen and Roholm (1939), Brown (1939), Blumstein (1938) and Valls, Ottolenghi and Schajowicz (1942).

Advantages of Aspiration Biopsy

The advantages of aspiration biopsy are clear :

(1) While it is generally admitted, on the one hand, that there is little risk in carefully punching out or removing by scalpel (surgical biopsy) a small portion of tissue from an exposed ulcerated surface (skin, nose, mouth, rectum, uterine cervix and even bronchus) for histological examination,¹ it is, on the other hand, generally accepted

¹ Marzloff (1923) found that 36.8 per cent. of 38 long-surviving cases of cancer of the cervix had had a diagnostic curettage several days before operation and Magensen and Stout (1942) found 49 per cent. 5-year clinical cures with biopsy as against 34 per cent. without; Paterson and Nuttall

with Ewing, that a surgical biopsy through normal tissues is fraught with danger because such a procedure is liable to break down local barriers to the spread of infection, and, in the case of neoplasms, to favour early metastases, local carcinomatosis (see Plate IV, Fig. 1) or fungation of the growth through the surgical wound.

Since, however, injury to normal tissues surrounding a lesion is minimal because of a mere needle puncture, which, after removal of the needle, is quickly sealed by retraction of the tissues (and probably by a thin cylindrical blood clot in addition), aspiration biopsy offers an ideal method of obtaining pathological material through normal tissues at negligible risk.¹

(2) The operation is comparatively simple, can be completed in a few minutes and is eminently suitable for outpatients or for patients à domicile, thus rendering unnecessary the admission of patients into hospital and the preparations and expense attached to the use of a surgical theatre.

(3) While failure to obtain positive evidence at a first attempt (which is one of the few disadvantages of the method) does not rule out the presence of a diagnosable lesion, where the clinical data strongly suggest the presence of such a lesion, the biopsy can be repeated several times if necessary, without undue risk, until satisfactory material is obtained (See footnote under Technique, Method I.)

(4) The indications for its use are wide and few lesions particularly of a neoplastic nature, which would otherwise remain undiagnosed, need remain without a diagnosis.

Indications for Aspiration Biopsy

(1) Enlarged lymph nodes :

In cases suspected of metastatic cancer removal of a lymph node for diagnostic purposes should never be attempted (see Plate IV, Fig. 1). Aspiration biopsy is the method of choice and rarely fails to yield positive evidence in such cases.

In cases suspected of Hodgkin's Disease, requiring differential (1939) have reported similar findings in accessible carcinoma of the tongue and lips

¹ Using this method Coley *et al* (1931) found no adverse effects in bone tumours, and Martin and Stewart (1936) observed no untoward symptoms or sequelae in 3,500 aspiration cases although such regions as the lungs, liver, spleen, prostate, orbital cavity and neck, had been repeatedly aspirated. They also record a group of 60 cases of cervical lymph node metastases, proved by aspiration, which were free of disease from 1 to 6 years following treatment by radiation

diagnosis from other adenopathies, the common practice of removing a lymph node for histological examination may be rendered unnecessary if a needle biopsy proves positive whatever the lesion.

In any case, it is always advisable to aspirate the selected node first to exclude metastatic cancer.

(2) Any palpable tumour lying deep to the surface and covered by normal tissues, particularly breast tumours where diagnosis of malignancy is in doubt, or where, although diagnosis of cancer is clear, radical amputation is contraindicated.

(3) Cases in which an ordinary surgical biopsy could not be obtained without a major surgical operation, particularly where such a major operation would be unlikely to allow of the successful removal of the tumour.

(4) It is the only method feasible in the case of deep-seated lesions (suspected of being neoplastic) within the thorax, cranium, other bones and even within the abdominal cavity.

In the case of bronchial cancer, however, aspiration biopsy should be reserved for cases in which bronchoscopy has failed to yield *positive evidence in spite of strong clinical indications*, and provided the risk of spreading bacterial infection has been minimised by appropriate chemotherapy.

Disadvantages : The main disadvantage of aspiration biopsy lies solely in the small piece of tissue usually obtained and the possibility that its structure may have been distorted (Fig. 18) and prove a strain on the patience of the pathologist. The pathologist should realise, that although the piece of tissue which he is asked to examine may be less than he would prefer, nevertheless any positive information he may give will be of great value to both the surgeon and the patient. The surgeon, on his part, must be satisfied with less than the usual detailed report.

Accidents : Christiansen (1942) reports puncturing the femoral artery but bleeding responded to pressure, without further incident. Ferguson (1930) admits having inadvertently punctured the bladder on several occasions while aspirating the prostate but without any untoward after effects. One of us (F. Ellis) observed the following complications: (1) In one case infection tracked from the tumour to the skin. The existence of the infection had been suspected and some of the aspirated material, sent for culture previously, yielded a growth of *B. Coli*. (2) In a case of suspected lung tumour, pus was obtained from a depth of 10 cm. in the chest from an unsuspected empyema and the patient developed signs and symptoms of bronchopneumonia from which he died a fortnight later. (3) A

case of superior mediastinal obstruction with, emphysema was aspirated through the chest wall. No blood was obtained but the patient a man of 60, became rapidly ill and died within half an hour. The cause was not suspected until too late, but was confirmed at post-mortem examination as being due to a tear in the emphysematous lung which spread from the point of entry of the needle, so that the patient's respiratory embarrassment was fatally aggravated by a rapidly developing pneumothorax. Emphysema and infection in the chest might be regarded therefore as contraindications to aspiration biopsy through lung tissue. Similarly the abdominal cavity must be approached with care and after reasonable assurance by palpation that there is no intervening intestine.

Technique

There are at present two methods in use :

1. The original method devised by Martin, H. E. and Ellis, E. B. (1930) utilising a 20 cc. Record syringe and 18-gauge (or wider) sharp-pointed hollow needle.

2. A drilling method based on a modification by Christiansen, H. (1940) of one devised by Kirschner of Heidelberg (1934), utilising a trephine-like flat ground hollow needle with sharply bevelled edges and provided with a suitable trocar, adapted to a drilling machine as used by dentists

In both cases the needle should be about 6 cm. long for the average lesion. For intrathoracic and other deep-seated lesions a longer needle is necessary.

Method 1 : The skin is cleaned as for any surgical procedure, and an intradermal wheal made with 2 per cent. novocaine at the site chosen for puncture. If the tumour is at some depth it is desirable to introduce the local anaesthetic down to the surface of the tumour and to leave the "anaesthetizing" needle in situ as a guide to the direction of the "aspirating" needle. A small stab incision is then made with a scalpel through the skin¹ and the aspirating needle introduced, with or without the syringe attached, down to the surface of the tumour which can easily be felt. It is very useful to fix the tumour (particularly small ones such as lymph nodes) with the thumb and forefinger of the left hand, or, in the case of tumours in the submaxillary region, with one finger in the mouth. To aspirate the prostate gland

¹ The stab incision through the skin is always advisable, otherwise the aspirating needle is liable to get blocked with a small piece of firm normal epidermis which eventually proves to be the only finding in the finished histological preparation (Fig. 9).

the needle is introduced through the perineum and the forefinger of the left hand introduced into the rectum as a guide. The approach to lung tumours must of course be guided by suitable skiagrams of the chest.

When the needle is down to the surface of the tumour the syringe is attached and the piston withdrawn to provide suction ¹ (obviously the needle must fit the syringe without leakage) and held with the forefinger against the end of the barrel. Then still "fixing" the tumour with the left hand the needle is pushed into the tumour as far as its estimated deep aspect. Still maintaining suction the needle is withdrawn for a distance of about 0.5 cm. and then pushed in again for 0.5 cm at an angle of about 30° to its previous direction. This cuts off the column of tissue already in the needle. Then, suction still being maintained, the needle can be withdrawn to the surface of the tumour and reintroduced as before, through the whole thickness of the tumour and the cutting process repeated. When this has been done two or three times the suction is released very slowly and the syringe detached from the needle. The needle is now removed and bleeding, if any, stopped by pressure through a piece of sterile gauze. The syringe is then filled with air, readjusted to the needle, and the contents of the latter expelled into a suitable container (a centrifuge tube for small quantities of tissue, or a flat-bottomed tube of wider bore for larger quantities of material such as fluid or blood). The syringe is finally filled with fixative and the syringe and needle used to wash down the sides of the container.

Practical Points of Value are:

(1) A fixative with a high enough surface tension not to froth and a low enough density not to allow the pieces of tissue to float in it is a great advantage. Suitable fixatives are Masson's alcoholic picro-formol and Formol Alcohol.

(2) If the tissue is hard to expel from the needle (e.g. cartilage or bone) it should be pushed out with a stylet.

(3) If much blood is obtained it is an advantage to have at hand a 3.8 per cent. Sodium Citrate Solution which can be added to prevent coagulation (although Patton and Patton (Personal communication to Martin and Ellis, 1934), as a routine, use 1-2 ml. of blood in a centrifuge tube into which to expel the aspirated material).

(4) If much blood or fluid is obtained this will be sucked up carrying with it into the syringe any solid tissue obtained. In such cases it is profitable to spend a few minutes in searching for and picking out pieces of tissue from the syringe and off the end of the piston by means of the tip of a scalpel. A number of pieces can often be scraped to the

¹ Failure to maintain suction while the needle is being manipulated within the tumour is probably the most frequent cause of failure to obtain sufficient tissue, for aspiration alone with the needle at rest is not sufficient to draw tissue into the needle.

exit of the syringe and then expelled into the fixative. This may be necessary even after the syringe has been rinsed out with fixative.

Method II • The principles of technique are essentially the same as in the first method, but the needle is of trephine type, and is introduced

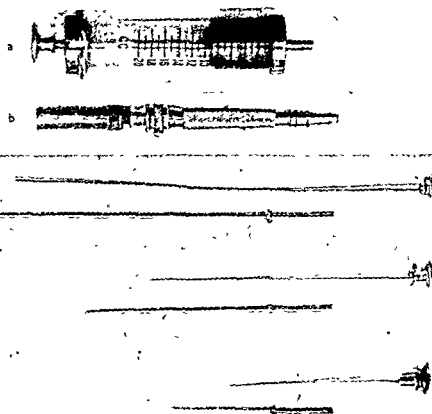


FIG. 10.—Essential parts of apparatus used by F. Ellis for drill biopsies. (a) 20 cc. Record syringe, (b) Chuck of dental drilling machine, (c, d, e) Three sizes of trephine needles with corresponding trocars. The needles fit into the chuck of the drilling machine during the drilling process and into the nozzle of the record syringe during suction and when expelling the contents of the needle into fixative.

with the help of a suitable trocar, and when down to the growth, the trocar is removed and the needle is attached directly to a dental drilling machine¹ so that the needle revolves as it is pushed into the

¹ F. Ellis has so far used an ordinary treadle drilling machine, but is of the opinion that a small portable electric motor as used and illustrated by Christiansen (1940) would render the operation easier and allow of simpler speed regulation.

lesion. It is to be noted that suction is unnecessary during manipulation of the needle within the lesion thus affording to the operator greater control than in the first method. When it is time to withdraw the needle for the cutting process, it is disconnected from the chuck of the drilling machine and the syringe is connected to provide suction during the cutting process.

The results with this method, in the hands of one of the authors (F. Ellis), have been so much more satisfactory that he now uses it exclusively. The advantages over the first method are:—

- (1) As already stated, the greater control afforded to the operator
- (2) The greater number of positive results secured, as shown in the following table:

RESULTS OF DRILL BIOPSIES * AS COMPARED WITH THOSE OF SIMPLE NEEDLE BIOPSIES

Regions	Simple Needle Biopsies		Drill Biopsies	
	Total No	Satisfactory	Total No	Satisfactory
Lymph Nodes	280	63 %	96	65 %
Breast	106	56 %	21	90 %
Bone	87	67 %	37	68 %
Lung	18	56 %	9	75 %
Intra-abdominal	13	64 %	10	80 %
Prostate	10	35 %	8	50 %
Total	514	55 %	181	71 %

Only biopsies from identical sources are given.

* All drill biopsies performed by F. Ellis at the Radiotherapy Department, London Hospital and reported on by Dr. Woods, Pathologist, London Hospital.

(3) The piece of tissue obtained is much larger, keeps its structure better and is more susceptible of confident detailed interpretation.

(4) While it can be used in all types of lesions, it is more suitable for hard lesions (e.g. fibromas, chondromas, osteo-chondrosarcomas, osteogenic sarcomas of sclerotic type and metastatic cancer of the vertebrae and other bones). For lesions within bone with a hard cortex it is advisable to introduce the aspiration needle through a hole in the cortex previously made with a bone drill worked by hand

Preparation of the Specimen for Histological Examination

Two methods may be adopted :

(1) The Smear Method as mainly employed by Martin and Ellis (1930) whose technique is as follows .

The fresh tissue fragment is transferred to a glass slide and smeared by firm flat pressure by means of another glass slide drawn once across. The smeared slide is fixed by heating gently over a gas flame until warm and dry, and is then prepared according to the following technique :

1. Alcohol (95 per cent.)—1 minute.
2. Water—1 minute.
3. Haematoxylin—1 minute.
4. Water—1 minute
- 5 Check by microscopic examination of the cellular stain, and if sufficient, proceed, or if insufficient, return to haematoxylin for deeper staining and then water—1 minute
- 6 Eosin—1 minute—check stain microscopically.
7. Alcohol (95 per cent.)— $\frac{1}{2}$ minute
- 8 Carbol-Xylol— $\frac{1}{2}$ minute
9. Xylol— $\frac{1}{2}$ minute
- 10 Mount with Canada Balsam and coverslip.

(2) The paraffin block method which the present authors prefer because the final histological sections are clearer and any available tissue better organised and susceptible of more confident interpretation. In the case of fluid material, however, whether clear or turbid, it is an advantage to examine a drop of it in the fresh unfixed condition between slide and coverglass in order not to miss such elements as cholesterol crystals from brachial and other cysts and cholesterol crystals and hooklets from hydatid cysts

For the paraffin method the material is placed in fixative as already described and sent to the laboratory. The method of dealing with the biopsy varies with the material available This may be

- (a) A small piece of tissue the size of a pin's head or several small pieces.
- (b) Loose cells suspended in some blood or fluid which impart a turbidity to the fixative.
- (c) A fairly large volume of blood with or without small solid pieces of tissue or loose cells. In this case the careful clinician may have done his utmost to pick out the small bits of tissue, in which case he will have sent two separate containers, one containing the bits of tissue and the other blood.

Procedure for Small Pieces of Tissue :

1. When there is no special urgency to issue a report :

Fixation is allowed to proceed for not less than 2 hours in the centrifuge tube.

Dehydration and embedding are secured by :

- Alcohol 70 per cent. 2 changes at intervals of at least 3 hours each
- „ 95 per cent. 2 changes at intervals of 1 hour each.
- Xylol 2 changes at intervals of $\frac{1}{2}$ hour each.
- Fresh Wax 2 changes at intervals of 2-3 hours each

The tissue is then picked off with fine forceps, embedded in fresh wax in a paper boat which is rapidly cooled in running cold water

The block is then sectioned and mounted in series so as not to miss any portion of tissue which may yield valuable histological information.

2. *When an urgent report is required* this can usually be issued in two hours by using the following technique :

From the outset the aspirated material should be transferred from the syringe into Masson's fixative which has the advantage of being an alcoholic fixative thereby reducing the time of dehydration. It is made up as follows :

Picric Acid	5 g.
Spirit	750 ml.
Formaldehyde	300 ml.
Glacial Acetic Acid	150 ml

Fix for $\frac{1}{2}$ hour.

Alcohol 95 per cent. 20 minutes.

Xylol (2 changes) 20 minutes each.

Paraffin (2 changes) 20 minutes each.

Block, cut and stain ($\frac{1}{2}$ hour).

Procedure for Fluid Material: This requires centrifugalisation after each step is complete, from fixation, through the alcohols (70 per cent. twice, 95 per cent twice) and xylol (twice) as above. After the two paraffin baths it is unnecessary to centrifuge the material, as it has now become coherent and remains so, but embedding must be done in the original centrifuge tube as follows :

After pouring off the second paraffin wax bath, leaving only about 1 cm at the bottom of the tube, the tube is very gently warmed from above to bring down all the wax which has by now solidified on its sides. This raises the level of the liquid wax to about 1.5 cm. Care must now be taken in handling the tube to ensure the material being embedded remaining at the very bottom of the tube and not to one side. It is rapidly cooled in cold water and when the wax has hardened the tube is wiped dry.

For removal of the block from the tube, the latter is very gently warmed from above downwards and at the same time rotated round its vertical axis to loosen the block from the sides of the tube. Great care must be taken not to melt the bottom of the block as the material embedded will otherwise immediately float up to the sides of the block (If this occurs the material will have to be taken back to xylol, spun again, and the embedding process repeated from that stage)

The tube is now quickly inverted to allow the block to come out. A sharp little tap on the bench may be necessary to detach it from the bottom of the tube (as the extreme lower end of the block was not heated to melting point).

Occasionally the block fails to detach itself and the tube must then be cut with a diamond low down below the level of the surface of the wax block and broken off. By means of forceps or the fingers, the block can be gripped and pulled out.

If the deposit of material at the bottom of the block is rather thick, the bottom end of the block while still warm and malleable is gently pressed with the fingers in the long axis to widen the cutting surface and so allow more tissue to appear in the section.

Procedure for Material mixed with a relatively Large Volume of Blood

After fixing in the usual way, it is centrifuged and the supernatant fluid poured off. With the help of a wire probe the material is detached from the sides of the centrifuge tube and expelled into a wider tube or jar (diameter depending upon the amount of the material), to allow the material to spread out and also allow the tissue to be more easily dehydrated. After passage through alcohol the material is usually coagulated, coherent and easy to handle. It is taken through Xylol to paraffin as usual, blocked in a paper boat, sectioned and stained in the usual way.

Staining: In most cases Haematoxylin and Eosin is sufficient. Occasionally Van Gieson's picro-fuchsin, Mallory's phosphotungstic acid haematoxylin and aniline blue connective tissue stain. Iron haematoxylin or Gomori's Prussian Blue method may be necessary and in the case of suspected lympho-sarcomas sections may have to be stained with a reticulin silver stain which is also extremely useful for demonstrating the architecture of necrotic tissues. If a search for bacteria is indicated sections may be appropriately stained by the Gram or Ziehl-Neelsen's method.

Interpretation: The interpretation of smears is much more difficult and more limited in scope than that of paraffin sections, and in our opinion does not make up in rapidity (which is claimed as the main advantage of the method) for what it loses in scope.

In the smear method, owing to shortened fixation and disorganization by the act of smearing, the cells appear larger and more loosely arranged than in properly fixed and sectioned paraffin sections and in general the interpretation is less certain.

of sections, the pathologist accustomed to paraffin sections finds himself contemplating a new

histology which he can only begin to understand after laboriously learning new criteria, particularly in the case of neoplasms, by making smears of unfixed specimens coming to the laboratory and comparing them with one another and with corresponding paraffin sections. While it is possible in this way to give reasonable broad interpretations of neoplastic material, particularly if guided by relevant clinical data, in the majority of cases, it is rarely possible by this method to determine the exact histological subvariety of a neoplasm and almost impossible to interpret inflammatory and other lesions except to exclude the presence of neoplasm.

Paraffin sections on the other hand, because of undisturbed organization, are definitely clearer and susceptible of more confident interpretation and in a great proportion of cases enable not only the diagnosis of the subvariety but even the histological grade of neoplasms, and the exact nature of many other lesions to be determined with accuracy as the appended illustrations clearly show. This is of great value to the radio-therapist.

The degree of detailed interpretation depends largely on the size of the material available, but also in the trouble taken in difficult cases (for example the use of differential staining methods where necessary) and on the experience of the pathologist, who naturally becomes more alert and more expert with practice. Much help in difficult cases can often be derived if all the relevant clinical data are provided by the clinician when the material is sent to the laboratory.

The authors have co-operated in the study of large numbers of aspiration biopsies from a wide variety of superficial but closed neoplasms and other swellings, with a very satisfactory proportion of useful positive results (70 per cent. of all cases).

When sufficient material is available a specific diagnosis can be returned in the majority of cases whatever the source, unless the tissue is badly distorted or necrotic.

Distorted tissue is usually unrecognizable (Fig. 18) but a good deal can sometimes be deduced from a small piece of necrotic tissue.

When the necrosis is due to tuberculous caseation it is usually complete and the material is finely granular and stains pink whether stained with haematoxylin and eosin or with Ziehl-Neelsen's stain; the granularity is also coarser than that of fibrin; a lympho-
 tubercle complete with giant cell system is present, ... infre-

quently tubercle bacilli can be demonstrated in sections stained by Ziehl-Neelsen's method (Figs. 12-15).

From lymph nodes containing metastases from squamous epitheliomas the material aspirated is in many cases necrotic, but more often than not the presence of varying numbers of keratinized cell-nests or pearls settles the diagnosis of a grade 1 squamous epithelioma. Such pearls sometimes show incipient calcification and in many cases are mixed with a polymorphonuclear exudate. In some cases a foreign body giant cell reaction is set up in the lymph node by the presence of these keratinized pearls, and the sections contain loose giant cells (Fig. 6). Sometimes, however, only loose squames mixed with granular albuminous material and blood is obtained and this may require diagnosis from the contents of a branchial cyst when the material has been obtained from the neck. In such cases the presence of polymorphonuclear leucocytes favours a diagnosis of squamous epithelioma; the presence of many lymphocytes on the other hand is more suggestive of branchial cyst as is also presence of cholesterol crystals in an unfixed wet preparation (Figs. 7, 8).

In portions of other necrotic neoplasms a definite architecture made up of masses of ghosts of cells separated by thin strands of collagenous or reticular tissue can often be recognized sufficiently to establish a diagnosis of malignant neoplasm though it may not be possible to diagnose the type (Fig. 16); in such cases the architecture is more clearly brought out by some silver impregnation method (Fig. 17), which may also enable some other lesion such as dead granulation tissue to be recognized. From material aspirated from lymph nodes it is usually possible to diagnose or exclude (in the tissue available of course, not necessarily in the remainder of the node) malignant metastases from a wide variety of primary growths. Other adenopathies such as inflammatory hyperplasia, simple lymphoma, leukaemia, lympho-sarcoma, reticulo-sarcoma and Hodgkin's disease present the greatest difficulty although it is sometimes possible to make an accurate diagnosis, but the exclusion of metastatic cancer is in itself, within limits, a valuable finding.

When the material available (whatever the source) is scanty, as for example several small masses or even a single small mass of about a dozen cells, a diagnosis of malignancy can often be returned with reasonable confidence based on such criteria as the unusual appearance of the cells, hyperchromatic staining, presence of mitotic figures (Figs. 2, 3), evidence of invasion of a different tissue

such as muscle (Fig. 4) and relevant clinical data, especially the exact origin of the material, for instance the presence of glandular acini of intestinal type in an abdominal scar (Fig. 5) or a single keratinized pearl from an obviously enlarged lymph node (Figs. 19, 20), etc. It is essential in such cases to examine as many serial sections as the block will provide, for while examination of the first two or three sections may contain little or nothing the next few that follow may yield the secret of the biopsy.

Failure to obtain any tissue is of little significance, and failure to obtain pathological material does not rule out the presence of a lesion. It is in fact the rule in the case of hard lesions such as fibromas and well-differentiated bony osteogenic sarcomas. It is worth remembering, however, that a negative result, in cases such as those just mentioned, far from being devoid of meaning may indeed be considered as evidence in favour of a reasonably confident clinical diagnosis.

Finally it may be mentioned that the search for parasites such as the plasmodia of malaria in bone-marrow, trypanosomes in lymph nodes, *Leishmania donovani* in bone-marrow, liver, spleen, or lymph nodes and *Leishmania tropica* in the local lesion of Oriental sore is best made in smears from puncture and aspiration of these organs and stained by Leishman's or Giemsa's method rather than subjecting the material obtained to histological examination.

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F. ELLIS.

PLATE IV

1. Carcinomatous invasion of the skin resulting from surgical biopsy of an invaded lymph node. The features are extremely pale and the area of local carcinomatosis mottled red and yellow.
2. Multiple small portions of an undetermined malignant growth aspirated from a cervical lymph node. $\times 65$.
3. Small portions of a squamous epithelioma aspirated from the sterno-clavicular region. $\times 100$.
4. Undifferentiated carcinoma invading muscle aspirated from swelling in neck. $\times 100$.
5. Adeno-carcinoma invading abdominal operation scar. $\times 100$.
6. Giant cell reaction in neighbourhood of cornified cell nest aspirated from cervical lymph node. $\times 100$.
7. Fluid aspirated from branchial cyst (paraffin section), showing loose squames and lymphocytes. $\times 100$.
8. Film of same fluid as No. 7 stained by Haematoxylin and Eosin showing cholesterol crystals. $\times 100$.

PLATE IV

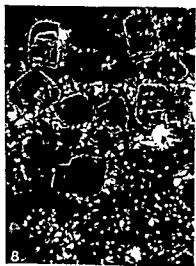
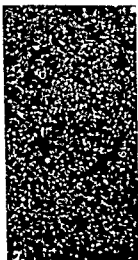
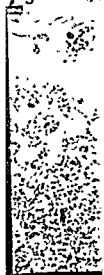
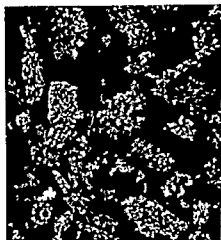


PLATE V

- 9 Piece of normal skin which has blocked the aspirating syringe
× 100.
- 10 Complete colony of *Streptothrix actinomyces* with surrounding pus
(sulphur granule) obtained by aspiration Gram Stain × 100
- 11 Part of the colony in No 10 (magnified).
- 12 Caseous tissue from cervical lymph node (T.B. +). × 100
- 13 Caseous tissue surrounded by more fluid material (T.B. +) × 10
- 14 Caseous tissue surrounded by reticulo-lymphocytic reaction
(T.B. -). × 100
- 15 Caseous tissue (top) and tubercle follicles with giant cell system
(bottom) T.B. + × 100
- 16 Necrotic carcinoma aspirated from swelling in neck. Type un-
recognisable × 100
- 17 Same tissue as No 16 stained by silver to show stroma × 100
- 18 Distorted tissue aspirated from cervical lymph node Unrecog-
nisable < 100

PLATE V



9.



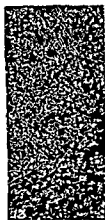
10.



11.



12.



13.



14.



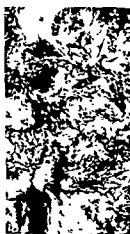
15.



16.



17.



18.

PLATE VI

The following (19-27) were all aspirated from metastases of squamous epitheliomas in cervical lymph nodes —

19. Single cornified pearl (Grade 1) $\times 100$
20. Single cornified pearl, loose squames and inflammatory cells (Grade 1) $\times 100$
21. Necrotic cornified pearls (Grade 1) $\times 100$.
22. Calcified squamous pearls $\times 100$.
23. Grade 3 squamous epithelioma. $\times 100$
24. Grade 3 squamous epithelioma. $\times 100$
25. Grade 2 squamous epithelioma. $\times 100$
26. Grade 2 squamous epithelioma. $\times 100$

PLATE VI

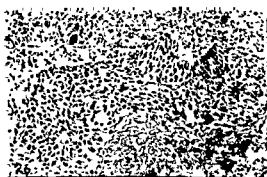
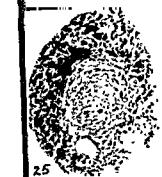
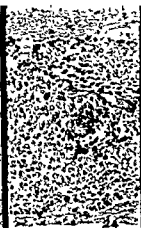


PLATE VII

27. Grade 3 squamous epithelioma. $\times 100$.
28. Basal cell cylindroma aspirated from growth of temple. $\times 100$.
29. Grade 3 squamous epithelioma aspirated from submaxillary swelling. $\times 100$.
30. Portion of neurinoma of spinal nerve aspirated from swelling near angle of jaw, and showing typical palissading of nuclei of Schwann cells $\times 100$.
31. Portion of aspirated cervical lymph node showing typical changes of Hodgkin's disease including several Dorothy-Reede cells. $\times 100$.
32. Melano-carcinomatous deposit aspirated from inguinal lymph node $\times 100$.

PLATE VII

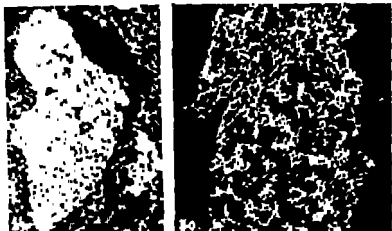
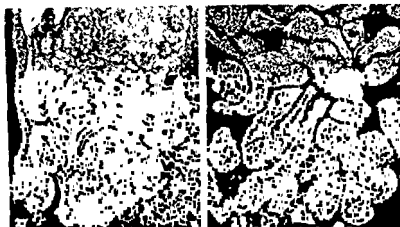


PLATE VIII

- 33 Granulation tissue aspirated from subacute inflammatory lesion of floor of mouth. $\times 100$.
- 34 Spindle cell fibro-sarcoma aspirated from calf of leg. $\times 100$
- 35 and 36 Pleomorphic fibro-sarcoma aspirated from pubis in a case of Paget's disease. $\times 100$.
- 37. *Pleomorphic fibro-sarcoma aspirated from biceps swelling.* $\times 100$
- 38 Muscle invaded by fibro-sarcoma aspirated from swelling of leg $\times 100$
- 39 Muscle invaded by an undifferentiated growth aspirated from groin. $\times 100$

PLATE VIII

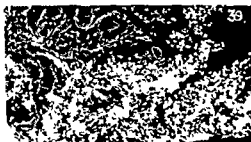
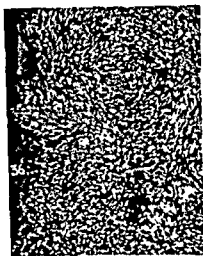
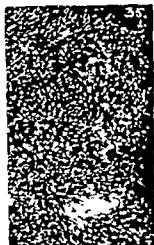


PLATE IX

- 40. Aspiration from myxo-chondroma of head of humerus $\times 100$
- 41. Aspiration from osteo-chondro-sarcoma of maxilla. $\times 100$
- 42. Aspiration from sacro-iliac osteoclastoma, $\times 100$
- 43. Plasma cell myeloma aspirated from sternum, $\times 100$
- 44. Loose plasma cells scattered in blood surrounding No. 43 $\times 255$
- 45. Myelocytic myeloma aspirated from upper end of humerus The cells belong to the granuloblastic series showing many mitoses and even young eosinophils. $\times 240$.
- 46. Secondary deposit of a well-differentiated adeno-carcinoma of the thyroid gland aspirated from skull, $\times 65$.
- 47. Metastases of breast carcinoma aspirated from sternum. $\times 100$

PLATE IX

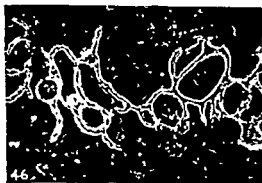
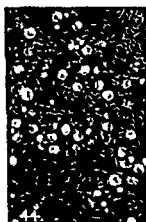


PLATE X

The following (48-52) are all aspirations from female breast swellings.

- 48. Mucoid (Colloid) Adeno-carcinoma. $\times 48$.
- 49. Scirrhus adeno-carcinoma. $\times 48$.
- 50. Anaplastic polygonal cell adeno-carcinoma $\times 100$
- 51. Medullary adeno-carcinoma. $\times 100$
- 52. Fairly well differentiated adeno-carcinoma. $\times 100$
- 53. Deposit of medullary adeno-carcinoma of breast aspirated from superior deep cervical lymph node. $\times 100$.

PLATE X

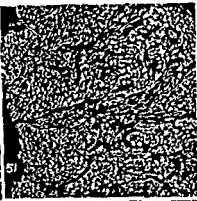
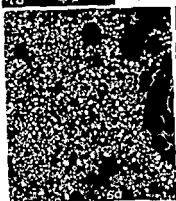
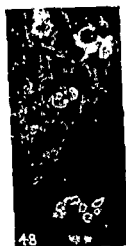


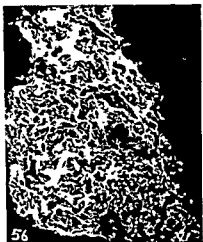
PLATE XI

54. Aspirated portion of normal submaxillary salivary gland. $\times 100$
55. Aspirated portion of normal parotid salivary gland. $\times 100$
56. Aspiration from mixed tumour of salivary gland situated in right upper eyelid $\times 100$.
57. Aspiration from an adeno-basal cell epithelioma below the right orbit. $\times 100$ from a patient who had been previously treated with X-Ray for a rodent ulcer of the nose.
58. Aspiration from mixed salivary gland tumour of submaxillary region. $\times 100$.
59. Aspiration from abscess of breast. $\times 120$.
60. Aspiration of liver, stained for iron. The patient had rather indefinite symptoms but showed a good deal of brown pigmentation of the skin, and the diagnosis lay between Addison's Disease and haemochromatosis. Presence of marked haemosiderosis in the liver settled the diagnosis.

PLATE XI



54



56



55



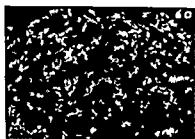
58



57



59



60

PLATE XII

- 61. Aspiration from upper lobe of lung. Oat cell carcinoma. $\times 100$
- 62 Aspiration from deposit of oat-cell carcinoma of lung in supra-clavicular lymph node $\times 100$
- 63.* Aspiration from adeno-carcinoma of bronchus $\times 100$
- 64, 65 and 66 Aspiration from three different cases of metastases from oat cell carcinoma of the lung in cervical lymph nodes $\times 100$

PLATE XII

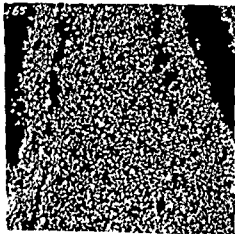
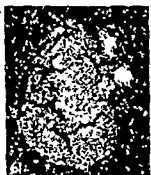
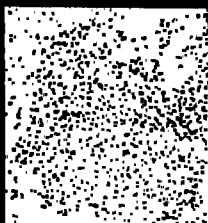
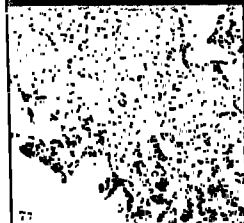
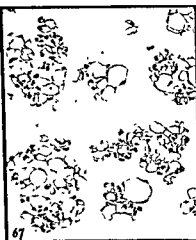


PLATE XIII

- 67. Section of deposit from fluid aspirated from the abdomen in a case of pseudo-mucinous adeno-carcinoma of the ovary. $\times 100$
- 68. Anaplastic adeno-carcinoma of uncertain origin (probably ovary) aspirated from swelling in groin. $\times 100$.
- 69. Aspiration from secondary deposit in liver of a Wilm's embryoma $\times 100$. Malignancy was diagnosed but the type of growth was only settled post mortem. It was an unusually cystic glandular one particularly in its metastases.
- 70. Aspiration from a primary adeno-carcinoma of the Thyroid Gland $\times 100$.
- 71. Aspiration from a seminoma of testis. $\times 100$.
- 72, 73 and 74. Peritheliomatous growth aspirated from cervical lymph node. $\times 100$. The suggested diagnosis was metastasis from a non-pigmented melanoma ($\times 100, 100$ and 200 respectively)

PLATE XIII



component cells. This apparent polymorphism is due to differences in the developmental stage of the malignant formations and is not incompatible with the conception of the essential homogeneity of the tumour tissue, *i.e.* its derivation from a normal parent tissue and its retention of restricted differentiation potentialities.

Young foci growing in favourable surroundings give remarkably similar counts from whatever part of the tumour they may be taken. The results of a quantitative analysis of selected areas in different peripheral parts of surgically removed tumours and of biopsies taken at intervals from the same area of untreated tumours are given in Table 1. The cells are classified according to their viability and the average percentage cell counts with their standard deviations for the different sections are recorded. The data show that reliable and comparable "samples" of young foci can be obtained by careful selection.

Counts of 200-500 cells per specimen should be made in a number of selected areas. If the counts are made at random in areas of different stages of development, no constancy in the results is obtained even if several thousand cells per specimen are counted.

In composite growths, such as skin tumours containing purely basal cell and purely squamous cell strains, or uterine carcinomata composed of adenocarcinomatous and of epitheliomatous tissue, young foci of each component have to be analysed separately and they will then be found to behave like homogeneous tissue.

Classification of Malignant Cells according to their Viability

Two groups of viable cells, (1) resting and (2) mitotic cells, and two groups of non-viable cells, (3) differentiating and (4) degenerating cells are classified. The resting cells form the "stock" cells of the tumour; they multiply by mitosis and if unable to divide they differentiate and/or degenerate. There is thus a constant progression of cells passing from one category to another.

(1) **Resting Cells**: Included in the term are the resting and the differentiating intermitotic cells (Cowdry, 1942). The former are undifferentiated cells varying with tumour type (basal, columnar cells, etc.) while the latter are cells which though differentiated (as for instance in merocrine or endocrine glands) are capable of division in a "resting" phase. Adenocarcinomata of various types contain such cells. The intermitotic period, *i.e.* the life span of resting

cells ranges from hours to days (Levi, 1934; Glucksmann, 1945) while that of the differentiating intermitotic cells ranges from days to weeks (Cowdry, 1942).

In any given tumour the resting cells are in the class of the smallest cells and usually have a relatively large, dark staining often hyperchromatic nucleus and relatively little, basophilic cytoplasm (Plate XIV, 1). These cells predominate in the younger parts of the untreated tumour and vary in their specific characteristics with the tumour type. Frequently polyploid variations of the smallest forms of resting cells are found either in separate strands or in the same area (Plate XIV, 2 and 3). These polyploid cells are proportionately bigger, *i.e.* their nucleus, nucleolus and cytoplasm are all enlarged and the latter tends to be more condensed.

In the more anaplastic types of growth a few very conspicuous mononuclear monster cells are seen. Since these cells may undergo mitosis or split up into multinucleate symplasms (Plate XIV, 6) by endomitosis, they have to be considered as viable and are included in the resting cell count unless they show definite signs of degeneration.

In adenocarcinomata cells attempting mucous or pseudomucinous secretion have to be considered as differentiating intermitotic cells unless they are actually undergoing mucinous degeneration.

(2) **Mitotic Cells:** This group comprises all normal dividing cells as well as slightly abnormal mitotic figures. Gross mitotic abnormalities causing death of the cell in division or the degeneration of both daughter cells are included in the degenerating cell count.

Prophase is the longest of all mitotic phases and is therefore most frequently encountered in normal and malignant tissues. The duration of cell division and of its various phases is subject to considerable variations. As a rule larger cells and malignant cells take a longer time to divide than smaller and normal cells. Slight or marked mitotic abnormalities tend to prolong the process.

The duration of the mitotic process (Levi, 1924; Lewis, 1939 and Mollendorff, 1938) in normal adult mammalian cells may be computed (in minutes) as follows: prophase 20-60, metaphase 4-24, anaphase 2-6, telophase 3-24. Taking as average a duration in minutes of 30, 14, 4 and 12 respectively, the percentage incidence of the phases can be reckoned as prophase 50 per cent., metaphase 23 per cent., anaphase 7 per cent., telophase 20 per cent. Counts made in 5 cases of human regenerating epidermis (following burns) and based on over 500 mitotic

metaphase percentage 22 ± 2.8 , anaphase percentage 9 ± 1.2 , telophase percentage 20 ± 1.8 . The incidence of prophase among the normal divisions for a number of tumours gives a percentage value of 57 ± 3.1 .

Mitotic abnormalities occur more frequently in the central parts of larger and older tumour strands. Their numbers increase with the mitotic index and with the degree of anaplasia of the tumour tissue.

The following values for abnormal mitosis (expressed as percentage of total mitotic count) in benign lesions and untreated tumours at various sites have been obtained :

	percentage
regenerating human epidermis (following burns)	11 ± 2.3
basal and squamous carcinomata of the skin	15 ± 1.4
epitheliomata of the tongue and oral cavity.	20 ± 2.4
epitheliomata of the uterine cervix	32 ± 3.3

Gross mitotic abnormalities (Politzer, 1934; Ludford, 1942) classified as degenerate cells are : the clumping of chromosomes in metaphase and anaphase leading to pycnosis; the clumping of chromatin in prophase resulting in hyperchromatosis of the nuclear membrane; the gross scattering of chromosomes at metaphase leading to chromatolysis. Bridge-formation in anaphase and spindle abnormalities resulting in multipolar divisions do not necessarily cause the death of both daughter cells and are therefore included in the mitotic count unless they are accompanied by scattering or clumping of the chromosomes.

(3) **Differentiating Cells** : These are cells rendered permanently incapable of division by the differentiation of their cytoplasmic structures. Their life span varies with tumour type but is usually of the order of days or weeks (Cowdry, 1942; Glucksmann, 1945). The most common feature in the differentiation of malignant resting cells is the process of parakeratosis (Borst, 1924) which begins with an increase in cellular, nuclear and nucleolar volume (Plate XIV, 1). The cytoplasm becomes more condensed and may form inter- and intracellular tonofibrils (Plate XIV, 1), the nucleus at first tends to become vesicular but after a temporary swelling of the cell, a stage in which the condensed cytoplasmic material is accumulated at the cell wall, both the cytoplasm and the nucleus shrink and the cell becomes a "squame" though it still contains the remains of a pyknotic nucleus (Plate XIV, 9). This process may

also in adenocarcinomata (Plate XIV, 4) and in basal cell tumours though with slight modifications. In the differentiating types of tumours almost normal differentiation processes are observed.

(4) **Degenerating Cells:** These vary in form according to whether mitotic, resting or differentiating cells are affected. Gross mitotic abnormalities and cytoplasmic changes resulting in cell death have already been considered.

Resting cells undergo various types of degeneration: (a) chromatopycnosis (Plate XIV, 10) begins with the appearance of coarse granules in the nucleus and crenation of the nuclear membrane, and proceeds to the shrinkage and pycnosis of the nucleus with subsequent chromatolysis, meanwhile the cytoplasm shrinks, liquefies or undergoes fatty degeneration. (b) Karyolysis (Plate XIV, 7, 8) starts with the disappearance of distinct nuclear structures and their replacement by a diffuse Feulgen-positive mass which subsequently shrinks and dissolves. The cytoplasm at first condenses (Plate XIV, 8) and then shrinks, liquefies or becomes fatty. This process occurs mainly in necrotic areas and in regions with an inadequate blood supply. (c) "Nucleolar" degeneration occurs mainly in mononuclear monster cells (Plate XIV, 5). The nucleolus or nucleoli increase disproportionately in volume and form a hyaline mass which at first often contains a central vacuole. The heterochromatin (i.e. the Feulgen-positive particles of the resting nuclei) becomes stratified and the nuclear membrane is dissolved allowing the nuclear contents to escape into the cytoplasm which at first condenses and finally shrinks.

The duration of the various degeneration processes (Strangeways and Oakley, 1923; Levi, 1934; Glucksman 1945) varies greatly according to their form and depends also on the rapidity of the resorption of the debris by phagocytes, etc. Subject to these variations, the duration of the various processes is of the following order: degeneration of mitotic cells lasts 1-3 or more hours; degeneration of resting cells about 7 hours; degeneration by parakeratosis or keratinization lasts days.

The application of the method to the analysis of localized, differentiating sarcomata is made by the use of a squared eye-piece. Cell counts in young foci are made per unit area and a decrease in cellularity due to the formation of bone, cartilage or fibres is taken as a measure of "differentiation." This method also allows of the measurement of uniform increases in cell size.

Changes Induced by Treatment: Changes induced in malignant tumours by radiation vary according to the tumour type and

PLATE XIV

Figures 1, 2, 3, 7, 8, 9 and 10 are taken from sections of untreated epitheliomata of the uterine cervix; figures 4 and 6 from sections of untreated uterine adenocarcinomata and fig. 5 from a section of a treated carcinoma of the nasal cavity.

1. Parakeratosis in stratified layers of a well-differentiated tumour
a = resting cell, b = differentiating cell. $\times 560$
- 2 and 3. Adjacent microscopic fields in the same tumour focus showing the differences in size of resting and differentiating cells in the two areas. $\times 400$.
4. Three successive stages (a, b, c) in the parakeratosis of columnar cells $\times 560$.
5. Nucleolar degeneration of mononuclear monster cells $\times 170$.
6. A multinucleate giant cell derived by endomitosis from a mononuclear giant cell. $\times 540$
- 7 and 8 Three successive stages (a, b, c) in karyolysis. $\times 1200$
9. Parakeratosis of individual cells (a) and subsequent stages (b, c) in the shrinkage and final resorption of the parakeratotic cells by neighbouring tumour cells. $\times 1200$.
- 10 Degeneration of resting cells: (a) chromatopycnosis, (b) hyperchromatosis of the nuclear membrane, (c) pycnosis, (d) chromatolysis. $\times 1200$.

PLATE XIV

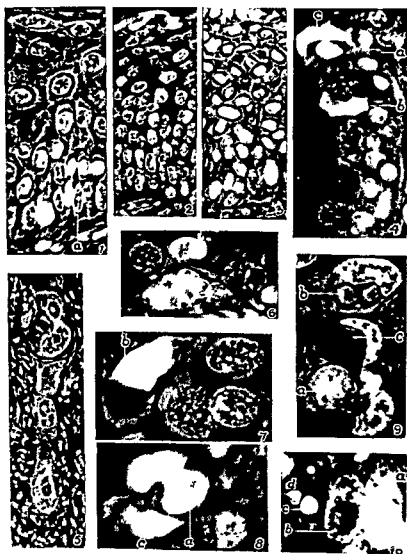


PLATE XV

Figures 1-4 show marked histological changes induced by radiotherapy in an epithelioma of the uterine cervix (see fig. 22).

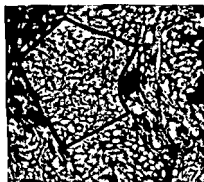
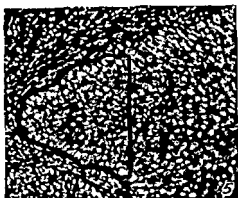
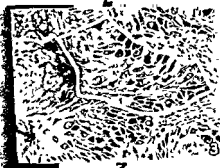
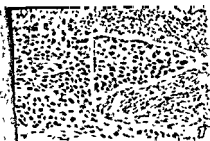
1. A young focus before treatment.
2. A young focus on the 2nd day of treatment
3. A young focus on the 7th day of treatment.
4. A young focus on the 11th day of treatment. $\times 75$.

Figures 5-7 show absence of significant histological changes in an epithelioma of the uterine cervix similarly treated by radiotherapy (see fig. 20)

- 5 A young focus before treatment.
- 6 A young focus on the 13th day of treatment.
- 7 A young focus on the 179th day of treatment. $\times 140$.

The outlined areas indicate the extent of fields chosen for cell counts

PLATE XV





the dose, dose-rate and time interval between a given dose and the biopsy excision. The aim of radiotherapy in malignant disease is to convert viable into non-viable cells, *i.e.* to induce the breakdown of dividing cells and to prevent cell division, to cause the direct degeneration of resting cells or their permanent sterilization by differentiation. The changes observed in the irradiated tumours follow from the direct effects of radiation on the malignant cells and the indirect effects due to vascular involvement.

The direct effects of radiation are seen particularly in the young foci and concern mainly resting and mitotic cells. After a transient mitotic inhibition, resting cells may break down on attempting division or may differentiate according to their type and potentialities. Some resting cells may degenerate immediately after exposure, but the dose required to cause the immediate destruction of all resting cells is far beyond the therapeutic level. Radiation frequently induces an enlargement of resting cells (Plate XV, 1-4).

After a period of mitotic inhibition cell division may be resumed at a normal or even a higher level and during this period of mitotic recovery the number of abnormal dividing cells may be greatly increased. Mitotic cells exposed to a sufficiently intensive dose of radiation become distorted and break down.

The direct effects of radiation thus diminish the number of resting and dividing cells and promote the "ageing" of cells and foci. The effect of radiation on cells in the early stage of differentiation has not yet been precisely determined.

The indirect effects of radiation are due to the interference with the vascular system and the induction of an inflammatory process. Insufficient blood supply affects the process and incidence of cell division and thus promotes the ageing of a focus. Marked vascular damage causes the degeneration (by karyolysis) of the most peripheral cells of a tumour formation, while cells, more centrally situated, are initially not involved and remain viable. Inflammatory reaction causes the break up of tumour strands by round cell infiltration and the scattering of viable cells.

Such scattered cells may cause a recrudescence of malignant growth; they should be distinguished from enlarged endothelial cells usually found in the vascular reaction. The differences in cytoplasmic structure of these two cell types are brought out very clearly in specimens stained with one of the modifications of Mallory's stain.

No difficulty is encountered in the selection of comparable areas in tumours which are responding unsatisfactorily to treatment (Plate XV, 5-7). In successfully treated cases, however, it may be

difficult to identify young foci in the later biopsies of a series. The selection of comparable areas in these cases is assisted by (a) the previous reaction of young foci to treatment, *i.e.* the progressive decrease in number of peripheral resting cells; (b) the lesser degree of round cell infiltration as compared with persisting "old" foci, (c) the presence of the regenerating epithelial surface which shows the biopsy to be taken from the "growing" edge. As in the evaluation of all biopsy findings, the presence of active foci carries considerably more weight than their absence.

Interpretation of Quantitative Findings: Cell counts made in selected areas of serial biopsies taken before, during and after treatment and plotted as percentages against time give graphs which fall into one of the following three groups:

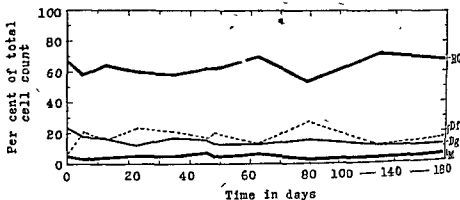


FIG. 20 —A graph showing only minor variations in cell counts thus indicating the persistent growth of the tumour and an unsatisfactory response to treatment (Plate XV, 5-7).

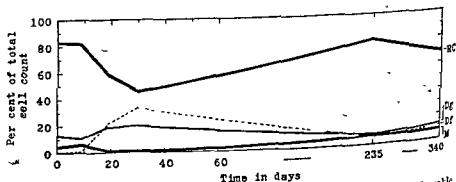


FIG. 21 —A graph showing a marked decrease in the percentage of viable cells but not their complete disappearance, thus indicating a temporary arrest of the growth followed by its recrudescence, *i.e.* a temporary response to treatment.

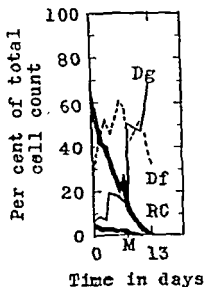


FIG. 22 —A graph showing the progressive diminution and final disappearance of all viable cells thus indicating a "local cure," i.e. the destruction of the tumour tissue in the treated area (Plate XV, 1-4).

The three graphs chosen as examples are taken from cases of patients with carcinoma of the cervix, treated by routine radiotherapy. The fate of the patients in these cases is as follows: (a) the growth persisted in spite of a temporary regression and caused the death of the patient 7 months after the beginning of treatment (fig. 20). (b) the growth disappeared temporarily but recurred in the treated area and caused the death of the patient 16 months after the

beginning of treatment (fig. 21); (c) the growth disappeared entirely and the patient is alive and free from growth 7 years after treatment (fig. 22).

Graphs of individual cases usually show the type of response to treatment within the first three weeks. The rapidity of the response varies to some extent according to the tumour type, treatment technique and site of the disease. The graphs indicate the reaction of the tumour tissue in the *treated area* only, and the biopsy findings can be regarded as typical for the whole treated region only if the dose delivered at the biopsy site is similar (i.e. within 10-20 per cent) to that given in all other parts of the tumour.

A favourable prognosis based on a response such as that shown in fig. 22, needs qualification if precancerous lesions are seen in later biopsies. Such conditions are frequently observed in luetic patients with carcinoma of the anterior portion of the tongue, in patients with leucoplakia of mucous membranes or in those with skin cancers arising in precancerous lesions (induced by pitch, tar etc.). Such precancerous changes may become malignant and invade the treated area.

In cases showing a temporary or unfavourable response difficulties in interpretation arise only very rarely.

Thus in two very exceptional cases of ulcerating basal cell carcinomata of the skin which progressed under treatment, graphs of type (a) were obtained. The lesions were subsequently excised and no evidence of malignant tissue could be detected in the operation specimen. The

ulceration by its round cell infiltration must have been sufficient to destroy the tumour (Ewing (1941)).

Application of the Method and Results : The value of histological tumour grading in surgical practice and in the evaluation of surgical results is now generally acknowledged (Taylor and Nathanson, 1942). Grading has not, however, provided any useful indication of the likely result of radiotherapeutic measures (Heyman *et al.*, 1941) owing to the fact that an examination of a pre-radiation biopsy gives no information concerning the developmental potentialities of a given tumour tissue.

The quantitative analysis of serial biopsies covering the period of treatment provides a means of studying the tumour potentialities and the extent of the reaction to radiation. Further, since histological changes in tumour activity become apparent clinically only after a considerable latent period, this method has some prognostic value in individual cases.

This may be illustrated by a comparison of the histological prognosis with the clinical findings subsequent to treatment in a series of 150 cases of uterine carcinoma (Glucksmann and Spear, 1945) shown in Table 2. In this series the histological prognosis

TABLE 2

Comparison of histological * and clinical evaluation of treatment results at intervals in a series of 150 cases of uterine carcinoma

	Months.				
	1-4	5-8	9-12	13-24	over 24
Agreements (in no. of cases)	82	104	108	111	111
Disagreements (in no. of cases)	68	42†	39†	38†	25†
No report (in no. of cases)	0	4	3	1	14

* The histological evaluation is made within three weeks of beginning treatment

† Includes nine patients known to have had insufficient treatment in affected regions at a distance from the biopsy site.

made within three weeks of beginning treatment was correct in about 80 per cent. of the cases. Disagreements between the histological and the clinical assessment of the therapeutic results were most marked in the early periods following treatment when the persistence of viable foci failed to cause clinical symptoms. In the series of Table 2, 58 such cases were observed in the period 1-4 months and only 26 in the period 13-24 months.

Similar results have been obtained in approximately 300 additional cases of tumours taken from various sites. It should be borne in mind, however, that the histological prognosis applies only to the reaction of the tumour tissue in the treated area. The patient may die of the disease as a result of distant metastases which were not treated, or because the damage to the vascular system in the treated area caused a fatal haemorrhage. In neither of these conditions, however, can death be attributed to the persistence of growth in the treated area.

Apart from its prognostic value in individual cases, the method can be used to obtain more accurate information about the effects of any given treatment on malignant tissue and about the biological changes produced by different treatment techniques or in different types of tumour. Such information provides a basis for attempts to improve the results of treatment by using the smallest possible dose to the greatest advantage, thus reducing the dangers attending overdosage; it may also indicate what additional therapeutic measures should be applied and the most opportune moment at which to apply them.

A. GLUCKSMANN.

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From the viewpoint of descriptive histology, it is much simpler to enunciate the main features by which the various lymphadenopathies can be recognized, but this presupposes that the diagnosis has already been made or else requires that the clinical histologist look at description after description until one is found that appears to match the node under examination. Here an attempt will be made to set out the deductive analysis by which the diagnosis may be made. In a normal lymph node the follicles are the structural units most easily recognized and so it is on their presence or absence that the commencement of the analysis may be based.

I. THERE ARE OBVIOUS NODULAR COLLECTIONS OF CELLS.

(A) The nodular structures are true follicles: If there are obvious follicular structures, it is essential to know whether these are true follicles—that is to say, the follicles normally found in lymph nodes, or "false" follicles—collections of cells arranged in a focal and circumscribed manner. The true follicles are chiefly situated at the periphery of the node and seldom at the hilum; they are evenly spaced and have a spherical outline; they are formed of closely set small lymphocytes with occasional large lymphocytes unless they are showing Flemming's centres; they may show a central arteriole, sometimes with hyaline change in the media; there is little or no reticulin in the follicle but a rim of reticulin demarks it from the medulla.

(a) The follicles are large and show Flemming's centres: The characters of the Flemming's centres have already been described (p. 352) and their presence is good presumptive evidence that the lymphadenopathy is a reactive condition, although a progressive hyperplasia may be superimposed on a reactive change. Accordingly it is necessary to analyse the changes in the medulla and sinuses.

In "*Reactive Hyperplasia*" (Fig. 1) there are prominent follicles with Flemming's centres and the medullary tissue is somewhat oedematous with separation of the cells and scattered amongst the lymphocytes are histiocytes and mononuclear reticulum cells; furthermore the endothelium of the venules of Schulze is prominent so that in cross section they may resemble epithelial tubules; there may be some degree of sinus catarrh (p. 358). *Reactive hyperplasia* is the characteristic lesion in the lymph nodes in the Still-Felty syndrome and on occasions the lymphadenopathy and splenomegaly

may precede the arthritis. In *Glandular Fever* a similar appearance is found but in addition to the reticulum cells and histiocytes there are numerous monocytoïd lymphocytes both in the medulla and follicles (Gall and Stout, 1940). In prodromal *measles*, there is a reactive hyperplasia, but in addition, both in the enlarged follicle and in the medulla, the characteristic Warthin-Finkeldey giant cells with bunched pachychromatic nuclei are present (Corbett, 1945). *Lympho-histiocytic Medullary Reticulosis* (Fig. 2) has a characteristic appearance. There are the changes of a reactive hyperplasia but in addition scattered irregularly through the medulla and occasionally in the follicles are small collections of mono- or multi-nuclear epithelioid cells, seldom more than two or three together and never showing caseation; a similar appearance may be seen in a very early tuberculous involvement of a lymph node but in tuberculosis there is almost invariably coalescence of the epithelioid system and small areas of caseation; in *sarcoidosis* (v. infra) the epithelioid systems are usually spherical and larger and the lymph node involvement is much more extensive; furthermore it is unusual in sarcoidosis for Flemming's centres to be present. In *Leishmaniasis* the histological change in the lymph node is that of a lymphohistiocytic medullary reticulosis, but the Leishman-Donovan bodies can be identified in the epithelioid histiocytes. In *Acute or Subacute Lymphadenitis*, there is a reactive hyperplasia but the cytology of the medullary proliferation is mainly formed of plasma cells with scattered collections of neutrophil leucocytes in the sinuses and around the blood vessels; sometimes the sinuses are markedly dilated, containing oedema fluid, numerous macrophages, histiocytes and leucocytes. In a *Chronic Lymphadenitis* there is a reactive hyperplasia, with a fibrosis of the periadenoid tissue and proliferation of chronic inflammatory cells in this zone; there may be some thickening of the trabeculae. Chronic lymphadenitis may be confused with Hodgkin's disease or vice versa, but if it is remembered that Hodgkin's disease seldom shows Flemming's centres and always causes irregular distortion of medullary tissue, the distinction between them should not be difficult.

(b) The follicles are small but are widely separated. This suggests a medullary proliferation and the analysis of these conditions is dealt with later (II B., p. 359).

(B) There is follicular pattern but it is due to the formation of "false follicles": A "false follicle" is a focal collection of cells arranged to form a rounded structural unit, but in which the detailed histology and cytology does not correspond to the normal;

metastatic melanomata, though the use of a silver impregnation will reveal melanin which is quite inconspicuous in the ordinary haematoxylin-eosin preparations. Secondary deposits of *sympathicoblastomata* (Fig. 13) are also liable to cause difficulty since the nuclei of these cells very closely resemble the lymphoblast and the sympathogonia (neuroblasts) may resemble small lymphocytes. In diffuse metastases of this type, the small foci of lymphoid cells are preserved and the whole of the medullary tissue is replaced by sheets of sympathicoblasts with pale staining nuclei; pseudo-rosettes are occasionally seen, but the characteristic feature is that the tumour cells surround acellular areas staining a homogeneous pale pink and in which no pattern whatsoever can be distinguished; with special silver methods (such as those of Cajal) it is sometimes possible to demonstrate neurites projecting from the cells into these areas.

The *reticulosarcomata* do not usually present any great difficulties in recognition when there is a diffuse involvement, and the points of differentiation have been dealt with earlier (p. 357).

(b) There is a focal medullary increase: Secondary deposits of reticulosarcomata and infective granulomata may all present as a focal medullary cellular increase but the lymphadenopathy characterized by this type of change is *Hodgkin's disease* (*lymphadenoma verum*) (Fig. 9). In its "classical" form, Hodgkin's disease is easy to recognize but in the early stages may be difficult; it is then that diagnosis is of paramount importance for it is only in the early stages when the lymph node enlargement is apparently limited to a single site that treatment can offer any hope of prolonging life and even then it is slender. The "classical" features have been admirably described and illustrated by Pullinger (1932). The lymph node is enlarged, the follicles are inconspicuous or obliterated and throughout the medulla there are foci, tending to coalesce, of primitive reticulum cells with a pale staining cytoplasm and sometimes a synplasma in which the large pale oval nuclei with a prominent nucleolus and fine interlacing chromatin threads stand out clearly, sometimes as a result of endomitosis (karyokinesis is not commonly seen) there are binucleate or multinucleate forms in which the nuclei are arranged as a mirror-image—the Dorothy Reed or Hodgkin giant cell (in fact the multinucleate cells were first described in Hodgkin's disease by Billroth (1858) and Andrewes (1901) gave the first good account of the histology). Surrounding and interspersed between the reticulum cells are fibrocytes, lymphocytes and granulocytes usually eosinophil, though neutrophil and basophil granulocytes may often be present while the cellular focus is en-

meshed in collagen. Histiocytes are not prominent, though histiocytic and dictiocytic multinucleate cells may be present. Areas of necrosis are sometimes to be seen and this is usually associated with numerous neutrophil leucocytes. The proportion of cellularity and fibrosis is variable and it is possible to grade the tissue change as cellular, sclerocellular and sclerotic, though the degree of fibrosis appears to bear no relationship to the survival period or radiosensitivity. In the earlier involvement these features are not so obvious and it is usual to find that much of the lymph node pattern has been preserved but there are scattered foci of reticulum cells, eosinophils, lymphocytes and fibrocytes without any coarse fibrosis; a feature which is almost diagnostic (provided the cytology is compatible) is a thickening and prominence of the capsule and fibrous trabeculae; sometimes this takes the form of oval or spherical masses of collagen around the blood vessels of the medulla. A diagnosis of Hodgkin's disease requires the disturbance of pattern and altered cytology and it must be recognized that multinucleate cells of the Dorothy Reed type and eosinophilia occur in many conditions both reactive and hyperplastic other than Hodgkin's disease. The condition which is liable to cause greatest confusion is a scarred node following a subacute lymphadenitis (see p 355), in this there is a considerable diffuse and focal collagen increase and there are often collections of eosinophils and reticulum cells. The fundamental distinguishing feature is that in a scarred lymphadenitis, the fibrosis is in relation to the obliterated sinuses whereas in Hodgkin's disease it is due to thickening of the trabeculae; follicles are commonly preserved in the first condition, and have disappeared in the second. Lastly the pattern of cellular increase is different in scarred lymphadenitis: the histiocytes and reticulum cells are in small foci of two or three surrounded by normal medullary lymphoid tissue, whereas in Hodgkin's disease the foci are larger, more polymorphic and there is a fibroblastic zone between the cellular area and the surrounding tissue.

And so one returns to the first criterion of histological diagnosis—real familiarity with the normal morphology and cytology of the tissue concerned and its possible variations. It is not imagined that this analytical presentation of lymph node diagnosis is infallible or foolproof—the first time one uses a key to a Flora, it is easy enough to identify a buttercup as an oak tree. However, it may give some guidance to those who see a limited amount of this material and stimulate thought and criticism from those who are working in the same field.

SUMMARY OF THE ANALYTICAL METHOD OF LYMPH NODE DIAGNOSIS

I. There are obvious nodular collections of cells in the node.

A. The nodular structures are true follicles (v. p. 354):

(a) *They are large and show Flemming's centres.*

Reactive hyperplasia; "glandular fever"; acute, subacute and chronic lymphadenitis; lympho-histiocytic medullary reticulosis

(b) *They are small and widely separated.*

Medullary proliferation (v. p. 359).

B. The follicular structures are "false" follicles (v. p. 355)

They are diffusely and evenly distributed throughout the node. Lymphoid follicular reticulosis; other follicular reticuloses; sarcoid.

C. The nodular cellular collections are irregularly arranged.

They are irregular in size and distribution; fibrocaceous tuberculosis; lymphogranuloma inguinale; metastatic deposits; reticulo-sarcoma; Hodgkin's disease.

II. Follicular structures are not obvious.

A. The sinuses are prominent.

Sinus catarrh, giant cell sinus reticulosis in children; chronic lymphadenitis with hyalinised sinuses; lipomelanotic reticulosis, metastatic carcinoma.

B. The sinuses are not prominent but there is a medullary cellular increase.

(a) *The medullary cellular increase is diffuse throughout the node.*

The leukoses, myelosclerosis, lympho-reticular medullary reticulosis, lymphadenoma verum diffusum; 'reticulum celled medullary reticulosis, the lipidoses; histiocytic medullary reticulosis.

Diffuse infiltration with secondary deposits of growth reticulo-sarcomata

(b) *There is a focal medullary cellular increase.*

Infective granulomata, secondary deposits of growth, 'reticulo-sarcomata; Hodgkin's disease (lymphadenoma verum).

SUMMARY OF THE NATURAL HISTORY OF CERTAIN OF THE LYMPHADENOPATHIES

The incidence figures are based on a six years' survey in Oxfordshire and it may be that the frequency in other countries or other parts of England is different. The survival rates are based on a six-year survey

of a thousand cases of lymph node enlargement referred to the Oxford Lymph Node Registry and indicate the proportionate survival at the date of the survey; in a rapidly fatal condition in which the majority of patients were dead at the survey date, the survival rates can be accepted as representative, but in more benign conditions in which a high proportion of the patients were still alive at the time of the survey, the survival rates are probably not so good as will be found when reviewed after a ten-year period; to give some indication of this the number of cases still alive at the survey date is in brackets given as a percentage.

Follicular Reticulosis

Lymphoid follicular reticulosis (Follicular lymphoblastoma)

Incidence: 4 per million. Mean age at onset: 47.

CLINICAL FEATURES: Generalized moderate lymph node enlargement often with spontaneous regression. Splenomegaly (30 per cent.). Rarely skin or bone deposits. Considerable liability to serous effusions or oedema of limbs. Sarcomatous change may develop (8 per cent.) at a late stage (8-12 years).

HAEMATOLOGY: No significant anaemia. Total leucocyte count normal or low; lymphopenia (less than 2,000 per cu. mm.) in 56 per cent; lymphæmia uncommon. Sternal puncture shows a gross excess of lymphocytes. Sedimentation rate normal.

TREATMENT. Radiotherapy. Splenectomy if enlarged spleen is inconvenient.

SURVIVAL RATE (when 80 per cent. still alive): 1 year 96 per cent; 3 years 58 per cent., 5 years 42 per cent; 10 years 10 per cent. Prognosis not so good in younger age groups (30-40) or where there is lymphæmia.

See Salm (1940), Baggenstoss and Heck (1940).

Sinus Reticuloses

Lipomelanotic Reticulosis (Dermatopathic lymphadenitis).

Incidence: 0.6 per million. Mean age at onset: 50. Marked male preponderance.

CLINICAL FEATURES. Generalized lymph node enlargement invariably associated with chronic skin condition usually an exudative type of exfoliative dermatitis. A small proportion (15 per cent.) show hepatosplenomegaly. Appears to be distinct from mycosis fungoides.

LABORATORY INVESTIGATIONS: Leucocytosis 78 per cent. Marked Eosinophilia 65 per cent. Occasionally show an abnormal sugar tolerance of the lag type. In the exudative form, there may be a marked reduction in plasma proteins.

TREATMENT. Symptomatic but unsatisfactory.

SURVIVAL RATE (when 74 per cent. alive): 1 year 78 per cent.; 3 years 52 per cent., 5 years 22 per cent.; 7 years 7 per cent.

See Robb-Smith (1944)

Sarcoidosis (Besnier-Boeck's Disease; Schaumann's Disease; Lymphogranuloma benignum).

Incidence: 6.6 per million. Mean age at onset: 32.

CLINICAL FEATURES: Pleomorphic. Generalized lymph node enlargement 74 per cent. Pulmonary change 37 per cent. Skin lesions 19 per cent. Iridocyclitis 16 per cent. Splenomegaly 12 per cent. Salivary gland involvement 7 per cent. Bone involvement 5 per cent. An essentially benign condition of slow progression while the sequelae of the iridocyclitis is the only lesion which is likely to be incapacitating though cardiac failure either from pulmonary fibrosis or myocardial involvement may develop.

LABORATORY INVESTIGATIONS: Tuberculin reaction negative at a dilution of 1:100, 90 per cent. Hyperglobulinaemia 62 per cent. Lymphopenia 62 per cent.

Sedimentation rate often raised when there is hyperglobulinaemia.

It is desirable that guineapig inoculation of the tissue should be carried out in every suspected case.

TREATMENT: Symptomatic. Radiotherapy has been claimed to be beneficial. Splenectomy if the enlarged spleen is causing inconvenience.

SURVIVAL RATE (when 97 per cent. alive): 3 years 100 per cent., 5 years 90 per cent.

See Scott (1937); Reisner (1944).

Medullary Reticuloses

Leukoses (Haemic Medullary reticulosis, the leukaemias).

Incidence 36.3 per million (myeloid 20 per million; lymphoid 15 per million; monocytic 1.3 per million).

CLINICAL FEATURES, etc.: The characteristics of acute and chronic myeloid and lymphatic leukaemia and monocytic leukaemia do not require description, but leukopenic myelosis and lymphosis are not so well known, though by no means infrequent.

See Wintrobe (1942), Forkner (1938); Scott (1939)

Myelosclerosis (myelofibrosis; myeloid megakaryocytic hepatosplenomegaly)

Incidence. 1.3 per million. Mean age at onset: 56

CLINICAL FEATURES: Mild symptoms of anaemia with gross splenomegaly and moderate hepatomegaly and lymph node enlargement. Radiological evidence of myelosclerosis may not be present until the later stages.

HAEMATOLOGY: Leuko-erythroblastic blood picture with thrombocytopenia. Erythrocyte fragility reduced. Sternal puncture may give the characteristic picture, but if fibrosis or osteosclerosis is present it may be necessary to perform a sternal trephination to obtain a specimen.

TREATMENT: Supportive and on no-account radiotherapy or splenectomy. Provided the patient is able to lead a reasonably normal existence, blood transfusions should be withheld unless there is severe anaemia. The patients commonly maintain a remarkable constant erythrocyte level at about 3.5 million and 70 per cent. haemoglobin and even if transfused will rapidly return to their own level.

SURVIVAL RATE (when 25 per cent. alive): 1 year 100 per cent.; 3 years 90 per cent.; 5 years 50 per cent.; 10 years 25 per cent.

See Churge and Wachstein (1944); Erf and Herbut (1944).

Lympho-histiocytic Medullary Reticulosis

Incidence . 0.6 per million. Mean age at onset : 33.

CLINICAL FEATURES: Apparently a benign condition characterized by unilateral painless cervical lymph node enlargement of slow progression; occasionally found in other sites but there is little evidence of progression from one site to another. No splenomegaly.

HAEMATOLOGY No anaemia, normal leucocyte count but there may be a slight lymphocytosis (up to 6,000). Normal sedimentation rate

TREATMENT The masses are radiosensitive, but often no treatment is necessary.

SURVIVAL RATE (when 95 per cent. alive): 1 year 95 per cent.; 3 years 45 per cent.; 5 years 22 per cent.; 10 years 5.5 per cent.

Lympho-reticular Medullary Reticulosis

Incidence 6.0 per million. Mean age at onset : 31.

CLINICAL FEATURES A slowly progressing disorder commonly presenting with lymph node enlargement at a single site, most commonly cervical and after this has been treated by surgery or radiotherapy, there will be a symptom-free interval of several years and then a recurrence at a different site. There is a liability for paraplegia to develop in the later stage due to dural involvement and sarcomatous transformation may take place after a course of ten to fifteen years.

HAEMATOLOGY Normal erythrocyte and leucocyte count; 30 per cent show a lymphopenia (less than 1,500 per cu. mm.). Normal sedimentation rate

TREATMENT: The masses are radiosensitive but are usually amenable to surgical excision.

SURVIVAL RATE (when 66 per cent. alive): 1 year 97 per cent.; 3 years 79 per cent.; 5 years 55 per cent.; 10 years 18 per cent.; 15 years 6 per cent

Reticulum Celled Medullary Reticulosis (Oberling's acute reticulosis of infancy; Letterer-Siwe Syndrome).

Incidence : 0.6 per million. Mean age at onset : (a) in children 5 ; (b) in adults 41.

CLINICAL FEATURES: *Two distinct syndromes:*

(a) *In Children:* An acute fatal pyrexial illness with generalized lymph node enlargement and hepatosplenomegaly, haemorrhagic manifestations are frequent and some defects may be observed in the long bones.

The duration of the disease is about three months, death being commonly associated with respiratory infection.

(b) *In Adults:* A chronic disorder characterised by lymph node enlargement and splenomegaly with severe anaemia and intermittent attacks of irregular pyrexia but without cachexia. A proportion of the cases present or develop symptoms of idiopathic steatorrhoea.

HAEMATOLOGY.

(a) *In Children:* Hypochromic anaemia; neutropenia; thrombocytopenia.

(b) *In Adults:* Severe orthochromic or hyperchromic anaemia with leucopenia (less than 5,000 per cu. mm.)

TREATMENT:

(a) *In children:* No effective treatment.

(b) *In adults:* Radiotherapy induces almost complete remissions of considerable duration. Symptomatic treatment of the anaemia and steatorrhoea.

SURVIVAL RATE (when 7 per cent. alive): (a) *In children* 2-3 months. (b) *In adults* 1 year 92 per cent; 3 years 58 per cent.; 5 years 25 per cent; 10 years 8 per cent.

See Wallgren (1940).

Storage Reticulum celled Medullary Reticulosis (Lipoidosis)

See Thannhauser (1940).

Histiocytic Medullary Reticulosis

Incidence: 2.0 per million. **Mean age at onset:** 42.

CLINICAL FEATURES. A rapidly fatal illness characterized by high irregular pyrexia, rapid wasting, gross splenomegaly but only moderate lymph node enlargement. Rapidly increasing anaemia, with haemorrhagic manifestations and occasional jaundice and skin deposits

M ochromic anaemia with
 nce of regeneration or
 lei the usual features of a haemolytic anaemia. Erythrocyte fragility
 normal or diminished

TREATMENT: Transient improvement with transfusions. Splenectomy has been performed with at least temporary improvement and as the condition when untreated is invariably fatal, is worthy of further trial

SURVIVAL RATE (none alive): 4 months

See Scott, R. B and Robb-Smith, A. H. T. (1939).

Hodgkin's Disease (Lymphadenoma Verum)

Incidence 23 per million. Mean age at onset 33

CLINICAL FEATURES Usually presents with unilateral cervical lymph node enlargement with slight signs of debility, spreading to other lymph node sites, mediastinal involvement, splenomegaly and periodic pyrexia. May present as pyrexia of unknown origin with minimal lymph node enlargement or with pulmonary symptoms. Spinal cord symptoms not uncommon in later stages. Pruritis common but skin involvement very rare

HAEMATOLOGY Slight degree of hypochromic anaemia. Neutrophil leucocytosis. Eosinophilia uncommon (85 per cent of cases under 500 per cu mm) Sedimentation rate commonly raised (75 per cent. cases over 50 mm in 1 hour)

TREATMENT Radiotherapy gives symptomatic relief but it is doubtful if it prolongs life. In early cases radical block dissection of involved lymph nodes appears to improve prognosis

SURVIVAL RATE (when 48 per cent alive) 1 year 64 per cent ; 3 years 19 per cent , 5 years 7 per cent. , 10 years 0.6 per cent.

See Middleton (1937)

Reticulosarcomata (lymphosarcomata, certain so-called "endotheliomata")

Incidence 12.5 per million Mean age at onset 46.

CLINICAL FEATURES May present as localized infiltrating mass of lymphoid tissue—inguinal region, nasopharynx or intestinal tract are common sites—or as generalized involvement of lymph nodes, liver, spleen, skin etc

HAEMATOLOGY Moderate hypochromic anaemia, with leuco-erythroblastic blood picture when there is bone marrow involvement

TREATMENT The masses are usually radiosensitive but response is transitory. Radical surgery where practicable gives much better results. There appears to be no correlation between the histological type (provided it is a reticulosarcoma) and radiosensitivity or prognosis

SURVIVAL RATE	No Special Treatment (12 % alive)	Radiotherapy (24 % alive)	Radical Surgery Radiotherapy (60 % alive)
	per cent.	per cent.	per cent.
6 months . . .	29	48	93
1 year . . .	6	15	75
3 years . . .	2	1.5	39
5 years . . .	2	1.5	10

See Edling (1938).

The observations in this chapter are based on a study of the material in the Oxford Lymph Node Registry which is supported by a grant from the Lady Tata Memorial Trust.

A. H. T. ROBB-SMITH.

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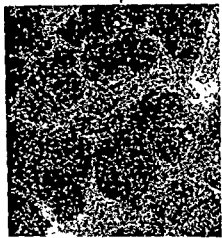
PLATE XVI

1. Reactive hyperplasia : four follicles at the periphery of the node all showing Flemming's centres. H E $\times 50$.
2. Lympho-histiocytic medullary reticulosis : a follicle showing a Flemming's centre is present in the lower part of the field and scattered throughout the medulla are small collections of epitheloid cells. H.E $\times 80$
3. Lymphoid follicular reticulosis. the "false follicle" is seen to consist almost entirely of cells of the lymphoblast series H E $\times 220$.
4. Lymphoid follicular reticulosis : the large "false follicles" can be seen to be compressing the medullary tissue. Reticulin impregnation $\times 50$
5. Sarcoidosis : there are numerous epitheloid "false follicles" sharply demarcated from the lymphoid medulla. H.E. $\times 70$
6. Hodgkin's Disease : there are nodular collections of proliferated cells lying in the dense fibrillary stroma. Reticulin impregnation $\times 50$.

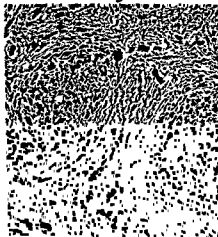
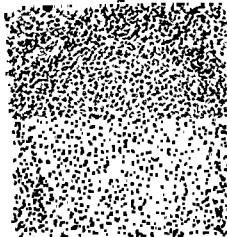
PLATE XVI



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PLATE XVII

7. Sinus catarrh: the dilated cortical and medullary sinuses are clearly seen. H.E. $\times 500$.
8. Lympho-reticular medullary reticulosis: there are scattered reticulum cells, some being multinucleate, lying in a lymphoid medullary stroma. H.E. $\times 220$.
9. Hodgkin's disease, an early stage in which follicles have been preserved at the periphery of the node, while the medulla shows the typical polymorphic cytology. H.E. $\times 50$.
10. Hyalinisation of the sinuses in a scarred lymph node: Note that the trabeculae are not thickened. H.E. $\times 50$.
11. Reticulum celled medullary reticulosis: there is a diffuse medullary increase of reticulum cells, some being of the histiocyte series H.E. $\times 220$
12. Lymphoid leukosis: there is a diffuse medullary increase of lymphocytes and the follicles are small and compressed to the periphery of the node. H.E. $\times 50$.

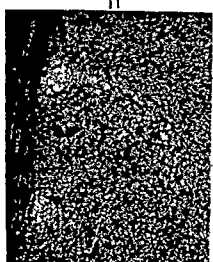
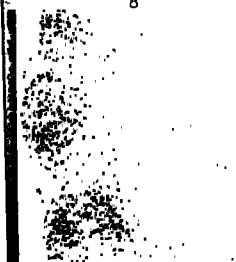
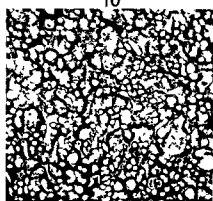
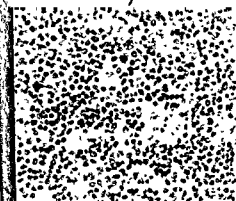
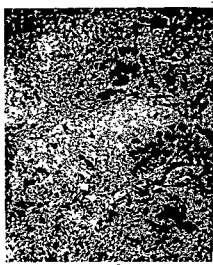
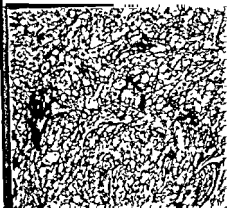


PLATE XVIII

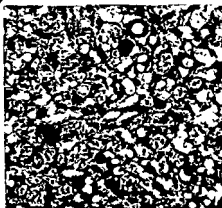
- 13 Secondary deposit of sympathicoblastoma: a follicle and surrounding lymphoid tissue is present, but the major portion of the field is occupied by sympathicoblasts showing the pseudo-rosettes and clear homogeneous zones around which the cells are arrayed H.E. $\times 100$.
14. Dictyosyncytial reticulosarcoma: the cells are isolated by an interlacing network of reticulin fibrils. Reticulin impregnation $\times 70$.
- 15 Secondary deposit of carcinoma: there are no reticulin fibrils separating the cells and there is a sharp demarcation between the sheets of carcinoma cells and the sclerotic stroma Reticulin impregnation $\times 70$
- 16 Dictyosyncytial reticulosarcoma: the cells appear to be in a synplasma, and the nuclear characters are typical of the cells of the reticular series. H.E. $\times 300$.
17. Secondary deposit of carcinoma: the cells showing a definite cytoplasmic membrane and the nucleus is leptochromatic with a dense nuclear membrane. H.E. $\times 300$.



13



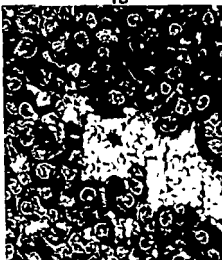
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CHAPTER XXXV

THE TESTICULAR BIOPSY

AZOOSPERMIA (absence of any spermatozoa from the semen) may be caused either by inactivity of the seminiferous epithelium or by defects of the ejaculatory apparatus (*e.g.* blockage of the ducts). Since examination of the semen does not usually allow differentiation of these conditions—both of which are common—direct examination of the testis may be necessary. This used to be carried out by puncture, *i.e.* by aspirating tissue, without incision, through the scrotal skin (Huhner's testicular puncture (Huhner, 1913)); the fragments thus obtained are usually unsuited for histological examination and more elaborate methods of biopsy were introduced (Lane Roberts *et al.*, 1938) and are now generally employed. Since these procedures yield tissue suitable for cytological study, the use of testicular biopsy has been much extended. It is now employed in the investigation of certain endocrine disorders (Heller and Nelson, 1945; Klinefelter *et al.*, 1942), in control of therapy of male sterility (Charny, 1940), and the study of testicular function in general (Hemphill *et al.*, 1944). In this review attention will be paid primarily to testicular biopsy in the diagnosis of male infecundity—the field in which it has established its value.

Indications

The indications for testicular biopsy are :

(1) Azoospermia; (2) Pronounced oligozoospermia in which it may be necessary to ascertain the condition of the tubules before treatment is attempted (see below); (3) Suspected endocrine disorders which may involve the testis. To this may of course be added cases in which malignant or other gross pathological changes, not related to fecundity, are suspected.

Testicular biopsy cannot be replaced, at present, by other methods of examination. Walker (1945) has emphasized that a clinical examination is not a reliable guide to the condition of the testes and the assay of fecundity. Fecundity is compatible with flaccidity of the testes; seemingly healthy testes may contain only degenerate or inactive tubules.

Technique—Experience has shown that it is difficult to render the puncture and exposure of the testis painless with local anaesthesia;

the latter is still used by some authors (Hemphill *et al*, 1944) while others advise a general anaesthetic (Walker, 1945). Where the testes seem to differ in size, turgor, or other respects, the seemingly healthier of the two is chosen for biopsy or specimens are taken from both. The testis is exposed through a 1 cm. incision; the tunica albuginea is punctured with a sharp-pointed tenotome and the small button of testicular tissue which presents itself is removed with iridectomy scissors. The tissue is transferred immediately to Bouin's fluid; even slight delay in fixation affects the structure of the spermatogonia and the spermatocytes.

- Paraffin sections are made; Haematoxylin Eosin or Haematoxylin Rose Bengal give preparations suitable for most purposes. Masson's stain, orcein and other stains have been used for studying hyalinization of the seminiferous tubules (Nelson and Heller, 1945).

Common Findings

Normal Conditions: The testis of fecund men is characterized by the predominance of active tubules. These have a thin membrane (tunica propria) and are lined by at least four concentric layers of seminiferous epithelium. While the spermatogonia and Sertoli cells are numerous the picture of the active tubule is invariably dominated by the large and numerous spermatocytes. Spermatozoa are often far less obvious, and while they may number several hundreds in a single cross-section, they may be comparatively scarce (20-50) in cross-sections through the tubules of fecund men. Some spermatocytes may show signs of degeneration (*e.g.* loss of nuclear membrane; pyknosis) but this is exceptional. Nor does the normal tubule contain many desquamated cells or much debris; exfoliation of incompletely differentiated cells is indeed *not* "normal." Multinuclear cells and lymphocytes may occur in small numbers. The head of intratubular spermatozoa does not usually show the differential staining (light anterior, dark posterior half) which characterizes the mature sperms found in fecund semen; the uniform staining, occurring within the testis, is not atypical or abnormal. The epithelium of the fecund tubule is rarely, if ever, arranged in that perfectly orderly manner represented in text books. Although most spermatides and the majority of spermatozoa are found within the central zone of the tubule, clear separation into concentric layers of spermatogonia, spermatocytes, spermatides and spermatozoa is not found and a certain degree of irregular arrangement, with the cell generations interspersed by each other is not only compatible with fecundity but represents the rule.

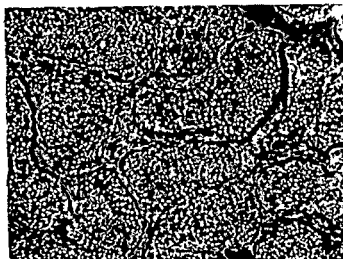


FIG. 1

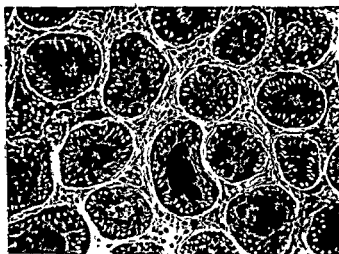


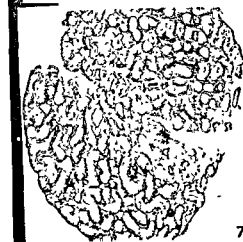
FIG. 2

1. Testicular Biopsy - Case of Azoospermia. Note moderate fibrosis of tunica propria, irregular outline of tubules, persistence of spermatogenesis of moderate intensity and occasional pycnosis.
- 2 Case of Azoospermia No history of orchitis or epididymitis. Note dedifferentiation of epithelium and fibrosis of tunica propria.

PLATE XX

- 3 Case of Azoospermia. Full differentiation of epithelium. The appearance of the tubules corresponding to the findings in some fecund and fertile men.
4. Case of Azoospermia. History of epididymitis. Dedifferentiation of epithelium with sloughing in some tubules and apparently increased volume of interstitial tissue.
5. Case of extreme Oligozoospermia. Prevalence of teratological forms, irregular outline and shrinkage of tubules, but persistence of tiered epithelium with differentiation ceasing at spermatocyte level.
6. Case of Azoospermia. Persistent differentiation, but interstitial odema
- 7 Case of Azoospermia. Fibrosis, absence of differentiation; occasional sloughing
8. Case of Azoospermia. Hyalinization.

PLATE XX



are either desquamated or degenerate, or fuse into multinuclear forms.

(2) *Atypical Exfoliation*: In normal tubules it is, generally speaking, only the fully formed spermatozoon that leaves the epithelial field; in abnormal tubules earlier cell generations are discarded with or without preceding degeneration.

(3) *Abnormal Cell Forms*: The secondary spermatocytes may undergo incomplete division and yield twincells (binucleate); conversely, spermatocytes and spermatides seem to fuse into multinuclear cells which pass into the semen. Other pathological cell forms include pyknotic spermatogonia and spermatocytes and, far more important than any others, partly differentiated, atypical spermatides and abnormal spermatozoa. These forms include not only the well-known types of abnormal spermatozoa which are commonly found in the semen (e.g. duplex forms; giant and micro-spermatozoa) but also spermatides in which the body of the spermatide has been preserved while a tail has been formed, etc.

(4) *Fibrosis of the Tunica Propria*: Lack of differentiation, atypical cells and desquamation are compatible with, and indeed are frequently associated with the persistence of a normal tunica. But fibrosis of the tunica is a common feature in the infecund testis and hyalinization is often its principal histological characteristic.

These types are to some extent independent of each other; moreover, the rate at which successive cell generations are formed and differentiate also varies greatly—from complete absence of mitosis to high frequency. Among the numerous types of tubules which may result, the following should be mentioned because of their common occurrence: (a) The epithelium is disarranged with some desquamated spermatocytes and spermatides lying in the centre of the tubule. A small number of spermatozoa may be present, either free or attached to the foot-cells. (b) the tubule is lined by a "healthy" epithelium but differentiation ceases at the spermatocyte level. Some of the spermatocytes have lost their nuclear membrane and the chromatin threads lie in the lumen. (c) The epithelium consists of several layers of cells including spermatozoa, but in the central zone, pathological cell forms are common and desquamation takes place. (d) The epithelium consists of two to four layers of spermatogonia and occasionally spermatocytes, but there is no lumen and the tubules have a small diameter. (e) The epithelium is reduced to a single layer of indifferent cells which fill the lumen with a network of plasma threads. (f) The tunica propria is thickened, the tubule is of irregular outline and small diameter.

(g) the tubules are represented by fibrous or hyaline cordons without germinal epithelium.

In *azoospermia* the testis may appear normal (predominance of active tubules); in milder forms of infecundity the testis differs from the normal merely by a change in the relative frequency of normal and abnormal tubules. It is only in the grave forms of infecundity that one finds a homogeneous tissue; in these the testis may consist entirely of inactive tubules or show uniform degeneration.

In addition to the condition of the tubules that of the interstitium should be considered. The interstitial tissue is often more prominent in infecund testes than in fecund ones. Experimental studies indicate that this apparent hypertrophy is often merely a reflection of the reduction in diameter of the tubules (Schinz and Slotopolsky, 1938), yet in our experience, infecundity is often accompanied by the formation of large aggregates of Leydig cells which may dominate the appearance of the section. Recently attempts have been made to utilize the cytological characteristics of Leydig cells in testicular biopsies for endocrinological studies, but the standards used are not yet established (Nelson and Heller, 1945).

Interpretation

The original—and still predominant—aim of testicular biopsy is the differentiation of testicular inactivity from blockage of the ducts; this is easily achieved. Where sperms are absent, blockage may of course exist as well, but its presence is of comparatively little importance in the absence of spermatogenesis. Where sperms are found in the testis but not in the semen a disturbance of the ejaculatory apparatus must be suspected. Recent findings (Barton, unpublished) indicate that this disturbance need not necessarily be a *mechanical one*—such as *atresia of the lumen of the epididymidal duct*—but may be functional. Thus some men may produce semen free of sperms during intercourse but ejaculate spermatozoa during nocturnal emissions or in masturbation. Accordingly the finding of normal testicular activity with *azoospermia* must be interpreted with due regard for the complexities of the sexual function. When biopsy has been carried out in men whose semen is not devoid of spermatozoa but contains only very few of them or a high proportion of abnormal forms, it is desirable to distinguish between cases likely to respond to treatment and those in which degeneration has advanced too far. Charny (1940, 1944) who has carried out

numerous biopsies in such cases states that in oligozoospermia the biopsy may show the presence of a few normally active, and of many completely degenerated tubules; the former do not require, the latter do not respond to, treatment. On the other hand, he and others hold that cases of oligozoospermia in which the tubules show a fairly uniform but rather low intensity of sperm-formation are suitable for treatment.

Since treatment consists quite commonly in the administration of gonadotropic hormones it would be desirable to have indications, in a biopsy of pituitary deficiency. In view of the findings in Rhesus monkeys and other animals (Allen, 1939) these signs might be expected to consist in a lack of spermatogenesis (with a tendency towards the retention of immature characteristics) but not in fibrosis or other pronounced degenerative processes or the formation of pathological cell forms. In clinical practice, however, testicular biopsies do not as a rule reflect uncomplicated or even evident pituitary failure. Gonadotropic therapy is only suggested by those biopsies which show low intensity of, or incomplete spermatogenesis.

In testicular disturbances for which gonadotropic deficiency cannot be held primarily responsible, interpretation must be in reference to the peculiar, well-established, but not generally considered sensitivity of the seminiferous epithelium to a great variety of noxious factors (Stieve, 1930; Allen, 1939). Among such factors are heat (the testis requires a lower environmental temperature than intra-abdominal organs, so that even a passing pyrexia may result in infecundity), many poisons (including some in industrial use); dietary deficiencies; X-rays; and bacterial toxins. Dietary deficiencies are possibly important but certainly never simple, thus neither biopsies nor clinical histories reveal testicular conditions which could be simply equated with the findings in experimental deficiencies (Mason, 1933) (Vitamins A, E). Toxic factors are probably much more frequently implicated. Thus it is significant that infective conditions are often associated with disturbances of spermatogenesis and that recovery from the former is often followed by restoration of the latter (Walker, 1945). Degeneration of the seminiferous epithelium is commonly found in men whose semen is rich in leucocytes and/or on culture yields pathogenic organisms. Distant foci of infection (e.g. nose and throat) are equally often associated with testicular deficiency. Unfortunately, it is not possible at present, to distinguish histologically between the various potential causes of degeneration of the seminiferous

epithelium, as they all seem to affect it in much the same manner as soon as a certain threshold is exceeded. It is only in the initial stages that, for example, damage caused by X-ray or Vitamin E-deficiency can be distinguished from each other or from other noxae (Schinz and Slotopolsky, 1924); non-hormonal factors should be suspected whenever the seminiferous epithelium is not simply at rest but exhibits signs of degeneration and/or pathological activity.

Before the physician can be recommended, on the basis of biopsies, to investigate such factors, he must be assured that the condition is likely to be reversible. It is therefore important to realize that even fairly advanced degeneration of the seminiferous epithelium does not preclude regeneration. In many animals such degeneration is seasonal; in man it is often found to be temporary, recovery taking place spontaneously or after treatment; recovery has never been recorded in cases of advanced fibrosis of the tubules even when the epithelium was partly preserved.

Interpretation of specimens relates, strictly speaking, to the testis from which they were taken; but in practice it is found that the testes differ but little from each other unless they show gross differences of size or firmness.

B: P. WIESNER.

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"hyperoestrinization," meaning the effect of an excess of the oestrogenic hormones. These oestrogens are sometimes called the "follicular hormones," because they are produced by the ovarian follicle during the first half of the menstrual cycle. Progesterone, similarly, is known as the luteal hormone, because it is produced by the corpus luteum. But in fact oestrogens are not only made in the ovarian follicle, and more progesterone is made by the placenta after the fourth month of pregnancy than by the corpus luteum.

The follicle ripens under the influence of the gonadotropic hormone of the anterior pituitary gland. The blood oestrogen level reaches a peak just before ovulation. The precipitating cause of ovulation is not known, but it seems that the follicle has to develop beyond a certain stage and that probably a small amount of progesterone is also necessary before rupture and the formation of a corpus luteum can take place (Novak, 1939).

During the second half of the cycle the blood levels of both oestrogens and progesterone reach a peak shortly before menstruation. Neustaedter (1939) points out that the corpus luteum produces even more oestrogenic substances than its precursor the Graafian follicle, and that during a normal cycle there is only a low level of oestrogens during menstruation. The precipitating cause of menstruation seems to lie with the behaviour of the endometrial blood vessels (Bartelmez, 1942) which in its turn depends to a great extent upon the sudden drop in the blood level of the ovarian hormones, particularly the oestrogens. A similar drop in the blood oestrogens may account for the phenomenon of endometrial bleeding occasionally seen at ovulation (intermenstrual bleeding).

While it is possible to measure the blood oestrogen levels, progesterone cannot conveniently be measured. Its presence can be inferred by the type of secretory endometrium found at biopsy, but it is doubtful wisdom to assume between these two factors a relationship which remains to be proved. Neustaedter (1939) has succeeded in producing the secretory phase in castrated women by giving sufficient oestrogens to cause tubular proliferation and by following this with progesterone, thus making secretory differentiation. On stopping medication bleeding took place. A big advance was made when it was shown that progesterone was converted into pregnandiol and excreted as sodium pregnandiol glycuronate in the urine. This substance is not difficult to measure (Randall, 1942; Wilson, Randall and Osterberg, 1939). Normal excretion rises from less than 1 mg. per 24 hours to between 3 and 5 mg. during the 12 days before menstruation, the peak occurring 6 days before with

low values for the last day or two. High figures are also found in pregnancy. Pregnanliol excretion is an unreliable method of measuring luteal activity unless repeated estimations are made. Moreover the endometrium does not necessarily correspond in its state of differentiation to the level of urinary pregnanliol measured. It has therefore been argued that the amount of secretory activity seen in the endometrium is a better guide to the luteal factors since instead of showing the amounts of these factors it reflects their physiological effect on the endometrium. This, as has been pointed out, is argument in a circle.

Abnormal Endometrial Patterns

These may either appear normal but at the wrong time in the cycle, or the appearance may be abnormal or both. The cause may be too much or too little oestrogen, or too little progesterone or to any combination of these.

Atrophic and Hypoplastic Non-secretory Endometrium: This is seen before the menarche, in castrates and in other cases of profound hypo-oestrinization. It is the only endometrial appearance which is invariably associated with one hormonal pattern.

Persistent Non-secretory Endometrium: When a non-secretory endometrium is found at a time in the cycle when secretory activity should have started (say within at least five days of menstruation) this indicates hypo-progesteronization and usually absence of ovulation, as in the non-ovulatory cycle. To confirm a cycle as non-ovulatory, however, it is necessary repeatedly to find a persistent non-secretory endometrium.

Hypertrophic or Hyperplastic Non-secretory Endometrium: In this condition, which is usually associated with high blood-oestrogen levels, there is great thickening of the tubular epithelium and some convolution of the tubules. Ciliation is often marked. The stroma tends to be fibrotic. The biopsy specimen is usually bulky. There is some argument as to whether there is true hyperplasia or only hypertrophy of the tubular epithelium. Herrell and Broders (1935) maintain that the process is hypertrophic; Kotz and Parker (1939a) that it is also hyperplastic. Probably the latter view is correct. Usually the blood level of oestrogens in this condition fluctuates rather sharply from time to time, producing irregular uterine bleeding. Fibroids are invariably accompanied by a hypertrophic endometrium, though sometimes secretory activity is also present. Sometimes when there are myometrial fibroids, fibro-

myomatous change may also be seen in the endometrial stroma. Rarely a hypertrophic non-secretory endometrium may undergo partial squamous metaplasia, without malignancy.

Cystic Non-secretory: Cystic changes in the endometrial tubules are indicative of ovarian failure, or hypo-oestrinization. Paradoxically they are usually accompanied by many areas of hypertrophic tubules. The explanation seems to be that owing to the fluctuation in the blood oestrogen level, sometimes hypertrophy and sometimes cystic changes are being produced. This state is usually accompanied by the clinical condition known as "metropathia haemorrhagica" (see Figs. 11 and 12). The curettings are

PLATE XXI

1. Early non-secretory endometrium. No history available.
2. Non-secretory endometrium. Age 20. Last menstrual period 16 days ago. Investigation for "sterility."
3. Non-secretory endometrium. Same case as Fig. 2.
4. Early secretory endometrium. Age 46. Para. 3. One month continuous bleeding. "Subinvolution." Partial hysterectomy.
5. Early secretory endometrium. Same case as Fig. 4.
6. Late secretory endometrium. No history available.

bulky and the cysts are usually visible to the naked eye. When castration takes place, an atrophic endometrium results. But this is not so at the menopause. Herrell (1939) has pointed out that at first there is luteal failure and the unopposed but variable activity of the oestrogenic hormones produces hyperplasia, followed by a varying degree of cyst formation when this too fails. The normal appearance of a post-menopausal endometrium is therefore cystic non-secretory, usually hypoplastic.

Delayed Secretory Endometrium (Fig. 13): Early secretory tubules may be formed either too late in the cycle, or mixed with hyperplastic or cystic non-secretory tubules. Fig. 13 shows a mixture of early and non-secretory endometrium. This delay in the appearance of secretory activity may be due to hyperoestrinization, to hypo-progesteronization, or to both.

Abnormal Secretory Endometrium: These may be (a) Hypoplastic, in which case there are either very few tubules with poor secretion and some cysts or else a normal number of tubules which are poorly differentiated. In this condition both oestrogens and progesterone are usually deficient. It may be difficult sometimes to distinguish a hypoplastic secretory from a non-secretory endome-

PLATE XXI

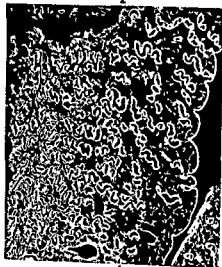
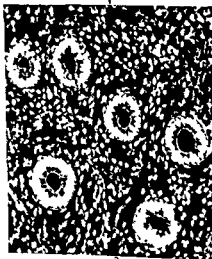


PLATE XXII

- 7 Late secretory endometrium Higher power to show pseudo-decidual cells in the stroma and secretory activity in the tubular epithelium
8. Menstruating endometrium. Age 38 "Menorrhagia" Period believed to be now present Regular dates
- 9 Early pregnancy. Age 40 Hysterectomy for fibroids. Chance finding of an early blastocyst with a presomatic embryo between twenty-two and twenty-eight days old.
10. Early pregnancy Same case as Fig 9 with higher magnification to show trophoblastic invasion of decidua.
- 11 Cystic non-secretory endometrium. No history available This appearance, sometimes called the "Swiss cheese endometrium," is typical of the clinical condition known as "metropathia haemorrhagica" The non-cystic tubules are hypertrophic
12. Cystic non-secretory endometrium The same case as Fig 11 at a higher magnification. The tubular epithelium is hypertrophic and obviously ciliated

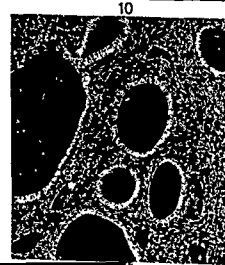
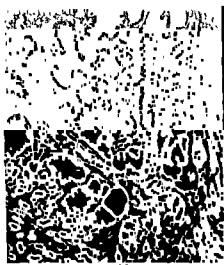
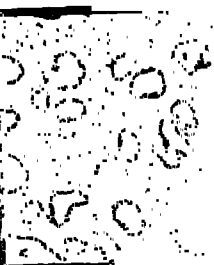


PLATE XXIII

- 13 Delayed secretory endometrium No history available
- 14 Late secretory endometrial polyp. Aged 41. Fibroids and endometrial polyp Hysterectomy Endometrial polyp usually show hypertrophic tubules, often with cyst formation and stromal fibrosis
- 15 Abortion with retained products Aged 38. Five weeks ago miscarried at four months
- 16 Adenocarcinoma Aged 60 Menopause ten years ago Two years menorrhagia
- 17 Chorion carcinoma Showing the hydatidiform villi from the uterine part of the growth.
18. Chorion carcinoma. Same case as Fig 18 Metastasis in vagina



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CHAPTER XXXVII

THE SKIN BIOPSY

DURING the past two generations dermatohistology has played an important role in the study of skin diseases, and pathological anatomy has exerted a profound influence on the shape of modern dermatology. The investigations were carried out almost entirely by the dermatologists themselves and such authorities as Jadassohn, Darier, Unna and Bloch were also expert cutaneous histologists.

In the present generation of dermatologists this has changed. New approaches to the complex problems of skin diseases were sought and eagerly pursued. This trend led inevitably to some decline in histological knowledge and the dermatologist now frequently appeals to the general pathologist for advice. The task that has fallen upon him is not an easy one and it is my aim to give him what little help I can in a short article.

There is no general rule as to when a biopsy is indicated as a promising means of investigation, and which of several lesions showing different stages of development should be selected. The dermatologist will improve as his pathological knowledge increases, he will learn to see clinically with a histological eye. If he cannot do the biopsy himself, he should at least choose and mark the place clearly; the choice should not be left to a junior assistant.

The technique of a biopsy

A biopsy is a minor surgical operation which every practitioner can perform in his consulting room. It needs no special instruments, but a very sharp knife is essential, one with a detachable blade (Bard-Parker, or similar make, size 15) is best. A blunt knife is bound to spoil the biopsy. Use 2 per cent. to 5 per cent. novocain with adrenalin as a local anaesthetic; adrenalin will hardly ever interfere with the histological picture. In small children a general anaesthetic may be needed. Shave the area if necessary. Avoid skin disinfectants that stain, such as flavine or iodine, or slippery substances like CTAB, but any colourless disinfectant, e.g. methylated spirit, will do. A towel with a hole in the centre which leaves the area of the biopsy free and covers the surroundings is an advantage. A biopsy should always include some of the normal skin surrounding the lesion, if possible at both sides of it. Try to arrange the excision along the cleavage lines; in the face, follow the natural folds and wrinkles. The routine shape of a biopsy is a strip of skin about 2.0-2.5 cm. long, 3-4 mm. wide, and deep

wool (Gurr) well in the oven, before weighing, and add it to Methylbenzoate in a wide-necked glass jar with a well-fitting glass stopper. It takes about one week to dissolve and should be well shaken from time to time. Once the solution is prepared it will keep indefinitely, if well stoppered.

Cutting: The secret of skin cutting lies in a sharp knife, obtained by very frequent stropping and only occasional honing.

My routine procedure is to cut ribbons of 12 sections, divide them, and place them on four slides, numbered in order, each carrying three sections. Cooling the block on ice makes cutting easier. Between each ribbon, stropping of the knife may be needed.

Staining. The four slides comprising one ribbon are stained as follows :

- Slide 1 Haematoxylin and Eosin.
- .. 2 Iron Haematoxylin and van Gieson.
- .. 3 Unna-Pappenheim's Methylgreen-Pyronin.
- .. 4 Acid Orcein (synthetic) and Polychrome Methylene blue.

Slide 2. van Gieson is preferable to Mallory's or Masson's stains, unless the sections are extremely thin. To examine the finest connective tissue fibres, use the van Gieson mixture alone, without previous Haematoxylin.

Slide 3. Methylgreen-Pyronin is an exquisite stain for cells, especially plasma cells. Technically, it is very simple, provided the tissue is fixed in Alcohol, or, better still, in Sublimate Acetic Acid (not Sublimate Formol). It is far too little used by pathologists, probably because the results are disappointing after Formol fixation.

Slide 4. I am convinced that synthetic Orcein (Conn) (Gurr's) will become the routine stain for elastic fibres in general pathology. It is one of the war substitutes that is better than the original, the natural Orcein.

The following are a few special stains that I favour :

Melanin. 1.5 per cent. Silver Nitrate, about 1 hour, faint counterstain with neutral red. Bloch's Dopa reaction, according to Laidlaw and Blackberg (1932).

Eosinophilic cells (Formol or Sublimate fixation) : stain faintly with haematoxylin, then with a very weak, watery solution of Eosin, (about 1 : 10,000) for 30-60 minutes. Result: Almost elective staining of eosinophilic cells, connective tissue practically unstained.

Iron : Gomori or Perl, very faint counterstain with neutral red.

Mucin : Thionin, according to Hoyer.

Amyloid : Methylviolet ; or the following modification of Congo Red (Freudenthal, 1930) 10 per cent. Formaldehyde 15-30 minutes, then directly into a solution of 1 per cent Congo Red to which 5 per cent. sodium carbonate has been added, 15 minutes, Aq. dest. 30 minutes. This modification is so sensitive

that it stains amyloid electively even if the Congo Red content of the solution is reduced to 1 : 100,000 or 1 : 1,000,000 ; it should then stain for 24 hours

Reticulin fibres : Robb-Smith (1937), or Laidlaw.

Xylol-Eosin : Useful for quick orientation during cutting. Place a paraffin section on a slide and add 1-2 drops of Xylol-Eosin. Within a few seconds the paraffin is dissolved and the section stained with Eosin at the same time. To prepare Xylol-Eosin, shake 5 cc of 1 per cent. aqueous solution of Eosin, to which a few drops of hydrochloric acid have been added, in 10 cc Xylol. Syphon off the now faintly pink Xylol. Xylol-Eosin keeps indefinitely.

Cutaneous histology covers a wide field. The normal anatomy and histology of skin is no small subject and before dealing with pathology it is advisable to acquaint oneself with the principal facts, I shall choose at random a few that have led to pitfalls.

The large mass of elastic tissue that forms the normal elastic cushion of the face has led to errors, especially if it is basophil in parts, as it often is in middle-aged and elderly people exposed to sunlight.

Sebaceous glands in the face may be very large and numerous without having any pathological significance. On the other hand the very small sebaceous glands attached to the scarce and fine lanugo hairs which are normally present on many parts of the body, especially of women and children, have been mistaken, even by dermatologists, as signs of atrophy.

Before reporting "pathological" changes of the vessels of the lower extremities, it is wise to study the normal conditions. One will find a bewildering variety in size, thickness of wall, arrangement of elastic fibres, endothelial lining, etc., even in people without any clinical signs of varicose veins.

Caution in general is advised when reporting on changes of the elastic fibres. They vary greatly, individually, and in different parts of the body. Moreover, their replacement, as is well known, may take a very long time and is often incomplete. Hence the lack or diminution of elastic fibres in a section may not be due to the present conditions, but may be a relic of a long forgotten boil or injury. Broken-up, curled, or diminished fibres may be residua of a stretching of the skin that occurred years ago owing to a rapid increase in weight, without producing clinically visible *striae cutis distensae*. Needless to say, similar changes are to be expected in the abdominal skin of women who have been pregnant.

Even seemingly unimportant details may cause trouble. In

normal sweat-glands, acid-fast granules of unknown significance may be found; these have been mistaken for granular forms of acid-fast bacilli.

Diagnosis

Most dermatological textbooks contain histological descriptions and pictures of the more common skin diseases. A list of textbooks on cutaneous histopathology is to be found at the end of this chapter. I shall confine myself to a few remarks on conditions which frequently cause difficulties to general pathologists and I shall add some notes on recent dermatohistological work.

Verruca senilis and Keratosis senilis: The seborrhœic wart (or Verruca senilis) and the Keratosis senilis (or Keratoma senile) are both common skin lesions which frequently give rise to histological errors.

The seborrhœic wart is found on the trunk, especially between the shoulder blades, also on the face and hands (Miescher *et al.*, 1936), the two latter localizations being also the common sites for Keratosis senilis. Histologically the seborrhœic wart consists of strands of more or less pigmented epidermal cells forming a network the meshes of which enclose horny cysts containing fine concentric lamellae, and connective tissue islands of a broadened papillary body. The width and length of the epidermal strands vary greatly; they may be long and narrow (Figs. 1, 2), reminiscent of sweat ducts, or short and wide containing 10-30 rows of cells. Often the strands become confluent and form solid epidermal masses. Sometimes these epidermal masses are more developed (acanthotic variety, Fig. 3), sometimes horny cysts and horny recessus are prevalent (keratoid variety). Occasionally, thick horny masses are heaped on top of the lesion, producing an appearance which one is almost bound to mistake clinically for a keratosis senilis or a prickle cell epithelioma. It must be admitted that histologically, too, the picture is sometimes deceptive, and reminiscent of an epithelioma, especially when long irregular epithelial proliferations are formed, (Figs. 1, 2). In spite of this, the seborrhœic wart—in contrast to the senile Keratosis—practically never becomes malignant. I am inclined to think that the seborrhœic wart is a tardive acanthotic naevus; there is hardly any evidence that it is related to the infectious common wart or to seborrhœa.

In Keratosis senilis the horny layer is thickened, parakeratotic areas alternating with hyperkeratosis at the orifices of the hair follicles and sweat ducts, where the rete cells stain darker than



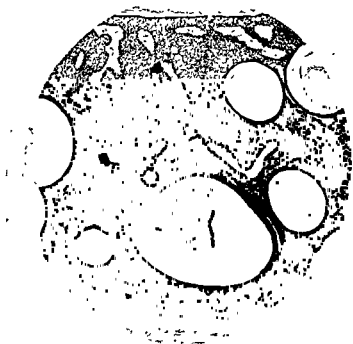
1

1. Verruca senilis (seborrhoeic wart), face. Horny layer greatly thickened, horny recessus and cysts filled with concentrically arranged horny lamellae. Very irregular atypical epithelial proliferation $\times 36$



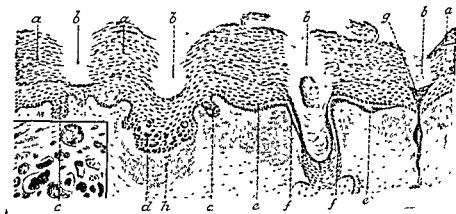
2

2. Verruca senilis (seborrhoeic wart), face. From lower left quadrant of figure No. 1. Atypical epithelial proliferation, cysts with horny lamellae, also horny "pearls" $\times 90$.



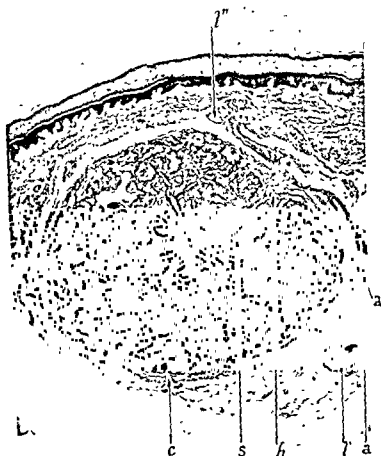
3

3. Verruca senilis (seborrhoeic wart of back). Akanthotic variety
Masses of epithelial cells, cysts with horny lamellae, strands and
islands of connective tissue $\times 60$



4. Keratosis senilis, semi-diagrammatical drawing. (a) parakeratotic pillars, (b) hyperkeratotic intervals; (c) whorls and buds, (d) clumping and giant cells (in left corner by higher power), (e) gap; (f) epidermal sheath around hair follicle; (g) darker stained funnel of sweat-duct; (h) Lymphocytic infiltration (W. N. Goldsmith, *Recent Advances in Dermatology*, p. 409, London, 1926)

PLATE XXVI



5. Glomus tumour. Round, sharply defined tumour consisting chiefly of glomus cells. Note fissures, half-moon and kidney-shaped spaces.

a, afferent artery, leading into the hilum, *h*, of the tumour, and narrowing into a slit, *s*. Connective tissue capsule, *c*, surrounding the tumour; *l'* and *l'''*, lamellar nerve corpuscles in its vicinity (Anderson, Weber and Freudenthal, *Brit. J. Derm.* 49 (1937) 151).

Pringle's disease in which, however, I have found as the most frequent alteration, fibromatous or fibroangiomatic changes (Roxburgh, 1945), sometimes of a peculiar character reminiscent of neurofibromatosis (Bamber, 1939, Davies, 1939); Butterworth and Wilson (1941) observed in their extensive material that fibromas are most commonly found; a hypertrophy of the sebaceous glands is considered an inconstant feature in Pringle's disease.

According to Savatard (1941) the rare sebaceous carcinoma may arise from the basal cells of the sebaceous glands or from the basal cells of the surface epidermis.

Naevus Mollis and Malignant Melanoma. Blue Naevus: I wish to call attention to two recent monographs, J. W. Dawson's (1925), and E. K. Dawson, Innes and Harvey's (1940), and shall confine myself to a few remarks.

It has been sometimes contended that a biopsy may precipitate or even provoke the change from a benign mole into a malignant melanoma, with disastrous results for the patient. I have never seen any ill effects in many soft moles excised, cauterized, or destroyed by other means. One should, however, take great care to ensure that one is dealing with a mole without any signs of beginning malignancy. If there is any doubt, a probative biopsy of part of the lesion, as in an epithelioma, is contraindicated, and complete excision with a fair margin of normal tissue is necessary, followed, of course, by histological examination. Bleeding of a mole is always an ominous sign. It is worth mentioning that warty or hairy moles on the upper part of the body hardly ever become malignant. It is the flat, pigmented mole, especially on the lower extremities, that is a potential danger. All the same, a malignant change is extremely rare, considering how common moles are. They are even more frequent than we imagine clinically, as is shown by the fact that in a biopsy a mole is sometimes found accidentally. What appears to be a freckle, may prove to be a naevus mollis when examined histologically.

Melanomas with their infinite variety of appearances give rise to diagnostic difficulties more often than any other skin tumour. The presence of melanin pigment, sometimes detected only after special stains (see p. 392), is certainly a good diagnostic guide, but it would be a grave error to rely too much on the absence of melanin in excluding a melanoma. Amelanotic forms are more frequent, especially in metastases, than is generally assumed. Pigment is often missing in the endo- and peritheliomatous varieties which I have seen chiefly on the lower extremities and which may be

mistaken clinically as well as histologically for angiomas or angio-endotheliomas. It is never easy, and sometimes impossible, to distinguish with certainty between a beginning melanoma and a naevus mollis which shows in parts and perhaps only temporarily, signs of activity. In making this responsible decision the clinical aspect should always be taken into account.

The Benign melano-epitheliomas of Bloch may clinically be mistaken for melanomas, but histologically they will be readily recognized. They are heavily pigmented ordinary epitheliomas, mostly of the basal cell type (Touraine, 1935). Conversely, the Blue naevus of Jadassohn-Tièche can in most cases be diagnosed clinically, but might histologically easily be mistaken for a malignant melanoma of the spindle cell ("melanosarcoma") variety. Like the Mongolian spot, the blue naevus consists of mesenchymal, melanin-producing, sometimes dopa-positive cells. They are mostly spindle-shaped, often with streamer-like prolongations, and are seen, more or less closely aggregated, mainly in the middle and lower part of the cutis, including the tela subcutanea; the epidermis is unchanged. Very frequently the tumour has a fibrous or fibromatous component. There is usually a good deal of melanin present, often distributed in irregular patches throughout the tumour, sometimes the pigment masses are so dense that the tumour cells are almost blotted out. The blue naevus is not nearly as uncommon as the small number of published cases suggest (Marquardt, 1937, Becker, 1930). It deserves to be widely known amongst pathologists. Malignant changes seem to be very rare (Darier, 1925, Bettley, 1938).

Cutaneous Metastases: The cutaneous metastases of malignant tumours of the internal organs are of practical and theoretical importance, yet the number of cases published is surprisingly small. O. Gates (1937) adds considerably to our knowledge by reporting and analysing nearly 100 cases, of which there is about an equal number of carcinomas and mesenchymal tumours. Further investigations in this line are desirable, a study particularly suitable for a general pathologist interested in dermatology. The scalp should be examined carefully as sometimes it is the predilected site, or even the only localization. In surgical cases I would suggest watching for spontaneous regressions, and investigating them histologically. Besides metastases, other cutaneous manifestations of malignant tumours deserve greater attention (Becker, Kahn and Rothman, 1942). It may sometimes be impossible to tell whether a single tumour originated in the skin or is a metastasis (Montgomery and Kierland 1940, Ronchese 1940).

Glomus Tumour (Masson): Subsequent to Masson's well-known research on the neuro-myo-arterial glomus (arterio-venous anastomosis of hand and foot), and its organoid overgrowth the glomus tumour, a considerable number of cases have been published. Since Anderson, Weber and I (1937) reported our first case (Fig 5) I have seen five more (one of them painless), in addition to the six cases (some of them atypical or doubtful) discussed by Twiston Davies, Hellier and Klaber (1939). The tumour cell ("glomus cell" or "epithelioid cell"), a large polygonal cell with a round or oval nucleus reminiscent of an epithelium cell was, in accordance with Masson's view, generally accepted as a modified unstriated muscle cell arising from the wall of the anastomotic vessel. Murray and Stout (1942), however, in their recent paper, suggest that the glomus cell is derived from the pericyte of Zimmermann which is widely distributed in the body and which would in the author's view, explain the occurrence of heterotopic glomus tumours.

Histiocytoma: The histiocytoma is clinically a small, hard, round or ovoid cutaneous tumour of greyish colour sometimes with a brown or yellow tinge and a rough, slightly hyperkeratotic surface, localized chiefly on the extremities. It was first described by Arning and Lewandowsky (1911) as "Nodulus cutaneus" and regarded as a fibromatoid granuloma. Woringer (1931) suggested that the tumour cells were histiocytes, and Seneor and Caro (1936) showed that they were able to store iron particles injected in their vicinity.

Histologically, it is a fairly sharply defined, non-encapsulated tumour, reminiscent of a cellular fibroma, consisting of numerous spindle-shaped cells and narrow strands of collagen tissue often arranged in whorls or interlaced ("fibroma en pastille," "dermato-fibroma," "benign dermato-fibrosarcoma"), (Fig. 6). Like the granuloma of the tendon sheaths the histiocytoma has a marked tendency to haemorrhages and to fatty infiltration. Lipoids may be found as fine droplets throughout the tumour, or in xanthoma (foam) cells, occasionally of the Touton variety ("fibroxanthosarcoma"). The haemorrhages result in a more or less intense haemosiderin pigmentation. The histiocytoma cell may, like the Gaucher cell, contain lipoid as well as haemosiderin granules. Occasionally these cells are arranged in nests, similar to those sometimes found in melanoma, but with a little care, easily distinguishable from them (Fig. 7). Arnold and Tilden (1943) found a family history of diabetes mellitus in nearly 50 per cent. of their cases.

Reticuloses : Mycosis fungoides, Hodgkin's disease, Kaposi's pigmented sarcoma, leukaemia, lymphocytoma and other diseases of this much disputed group are critically reviewed by Goldsmith and Robb-Smith (1944) in their papers on "reticuloses."

Mucin in Granuloma annulare. Histologically Granuloma annulare forms round or oval foci of more or less degenerated connective tissue surrounded by fibroblasts and epithelioid cells, often in a stellate arrangement. Mucin is always (Prunty and Montgomery, 1942), or very frequently (Freudenthal, 1945) present. It is found chiefly in the foci, the amount varying from traces to considerable masses. These findings are of theoretical interest as well as of practical help in the histological diagnosis of this disease, the aetiology of which had been much discussed in recent years. It might be worth while to search for mucin in the rheumatic nodule which presents a histological picture very similar to that of Granuloma annulare (Collins, 1937).

Familial Benign Pemphigus, etc. : A familial, chronic bullous dermatosis, localized mainly on the neck, axillae, groins and perianal region, has recently been described in the American literature and is generally regarded as a new clinical entity, though its classification is still under discussion. Pels and Goodman (1939) consider it a bullous variety of Darier's disease, Hailey and Hailey (1939) place it in the pemphigus group, and Ayres and Anderson (1939) near dermatitis repens. The controversy wages chiefly around the interpretation of the histological changes, the main features of which are intraepidermal ("acantholytic") fissures and lacunae accompanied by a proliferation of basal cells. Dyskeratotic cells may be found, though fully developed "corps ronds" or "grains"—characteristics of Darier's disease—are very scarce or entirely absent. Thus some authors prefer to look upon it as a separate dermatosis. (Dyskeratoid dermatosis, Frank and Rain, 1942; Dyskeratosis bullosa hereditaria, Becker and Obermeyer, 1940).

Value of the Biopsy

It is generally agreed that the skin biopsy is of considerable practical value. (Snedden, 1936; Montgomery, 1938) in the hands of Twiston Davies, finds that in only 43 cases were they of no help, in the rest, i.e. in nearly 80 per cent. he found them of value in one way or another. My own material does not lend itself to an accurate assessment, as a high

proportion is sent to me from sources of widely varying clinical experience, but I would put the figure at 50-60 per cent.

W. FREUDENTHAL.

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CHAPTER XXXVIII

THE PERIPHERAL NERVE BIOPSY

PATHOLOGICAL change in the connective tissue and vessels of a nerve trunk can adequately be demonstrated by routine histological methods. But since the condition of the axons and myelin of the nerve fibres is more elusive, special techniques must be added to the laboratory routine when specimens of peripheral nerve are to be investigated. Two such techniques will first be described, one for the myelin sheaths and one for the axons; they are applicable to formal material, and give controllable and constant results; the sections are cut by the paraffin method.

Technique

Myelin Sheath Stain on Weigert's Principle: The specimens having been fixed for three days or more in saline formaldehyde those portions are selected in which it is desired to demonstrate the myelin sheaths. Transverse slices from a large nerve trunk should not be more than from 3-8 mm. in thickness, but small nerve trunks such as the normal radial can be treated as a whole in pieces of any length. The selected pieces of tissue are placed, on the morning of the first day, in closed vessels containing many times their own volume of the mordant:

Chromium fluoride, powdered	1 g.
Potassium dichromate, crystals	3 g.
Distilled water	100 ml.

(solution can be hastened by placing the mixture in the 37° C incubator; the mixture keeps well)

The vessels containing the tissues and mordant are placed in the 37° C incubator and left there until late on the third day, that is, till after about 55 hours' treatment. The mordant is then poured away, and replaced by 70 per cent spirit. The tissues remain in spirit overnight, and on the fourth day are carried through the alcohol grades into the clearing agent (chloroform has usually been used). On the fifth day they are finally cleared in one or two further changes of chloroform. The last change may be kept perfectly free of alcohol and water by the presence of a few fragments of calcium metal in the stock bottle and dehydrating jar.

On the evening of the fifth day the tissues are placed in a saturated solution of wax in chloroform at 37° C, and remain in this in the incubator overnight. On the sixth day impregnation with wax is completed in the paraffin oven, and the pieces are embedded. Wax of a melting point of 57° C. has been found best for specimens of peripheral nerve

Sections are cut at about 6μ , usually transverse to the long axis of the nerve, since differentiation of longitudinal sections is more erratic: they are mounted by albuminization and dried overnight at 37°C . On the following day the slides are placed for an hour in the paraffin oven to complete the flattening and drying.

Some sections are reserved for trichrome staining. Masson's light green method, described by Foot (1933), is excellent, but for all formol material, whether mordanted or not, the diluted ponceau-fuchsin stain should be applied for a few minutes only, since otherwise it is impossible to obtain adequate differentiation during the phosphomolybdic acid treatment. The light green should be rapidly differentiated in 70 per cent. alcohol.

Sections of chrome-mordanted tissue can be successfully stained by other methods, such as Verhoeff's technique for elastic fibres.

The slides reserved for myelin staining are taken down to distilled water and placed in Kultschitzky's haematoxylin:

Haematoxylin, 10 per cent. sol. in absolute alcohol	10 ml.
Distilled water	90 ml.
Glacial acetic acid	2 ml

The 10 per cent solution of haematoxylin should be a month or two old, but the mixture above can be used repeatedly for weeks or months.

The slides remain in the haematoxylin solution at 37°C until any convenient time on the following day. Then proceed as follows:

1. Wash in running tap water, 5 minutes.
2. 0.25 per cent. aqueous potassium permanganate, 30 seconds—1 minute. (The permanganate may be kept as a stock at 2.5 per cent, diluting it ten times before use.)
3. Rinse off the permanganate in tap water, then in distilled water.
4. Treat for 10 minutes with a mixture of equal volumes of 0.5 per cent aqueous solutions of oxalic acid and potassium sulphite (The solutions should be mixed immediately before use the stock sulphite solution keeps for a few days only, but the oxalic acid indefinitely) While in the oxalic-sulphite mixture the slides should be examined at intervals under the staining microscope, as differentiation takes place progressively. When it is complete the myelin is black against a very pale brown or colourless background; the nuclei should retain little or no brown colour. When a mixed batch of slides is being stained a representative from each group should be examined at each stage.

Differentiation will probably be very incomplete after the first ten minutes of oxalic-sulphite treatment. In this case go back to stage 2 and repeat the permanganate treatment and differentiation and again, if necessary, a third and fourth time.

5. Rinse in distilled water, wash in tap water for 10 minutes, rinse in distilled water again
6. Counterstain in de Groot's iron carmalum for 3-5 hours at 37°C. This stain is prepared as follows :
Dissolve 0.1 g of ferric alum in 20 ml. distilled water ; add 1 g of carminic acid. Dissolve, add 180 ml. distilled water, warm, add 5 g. potassium alum, dissolve, cool, filter, add 2 drops strong HCl.
This staining solution can be used repeatedly and keeps indefinitely. The growth of moulds is prevented by adding a few crystals of *p*-dichlorobenzene to the solution.
7. Rinse in distilled water, dehydrate and cover. Results as in figs. 2, 4, 6 and 7.

Axon Stain, modified from Holmes (1943) : Special solutions required :

(1) Buffer solutions :

(a) Boric acid (A.R)	12.4 g
Distilled water	1000 ml.
(b) Borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, A.R.)	19 g.
Distilled water	1000 ml

(2) Reducer

Hydroquinone	1 g.
Sodium sulphite, crystals	10 g.
Distilled water	100 ml.

(the reducer can be used repeatedly, but may not keep for more than a few days).

Procedure :

The blocks of tissue are embedded *without mordanting*, longitudinal sections are usually the more informative and are cut at about 8 μ , though for some purposes, as in the study of end-organs, thicker sections are necessary. They are mounted and dried as for the myelin method ; some are reserved for Masson's light green trichrome and other stains.

For axon staining proceed as follows :

1. The slides are taken down to distilled water and placed in a 20 per cent aqueous solution of silver nitrate in the dark at room temperature, where they remain for 1 hour (up to 2 hours is permissible : the solution may be used repeatedly so long as it does not contain a black deposit of silver).
2. Meanwhile prepare the impregnating solution, which can be used once only. Take 55 ml. of the boric acid buffer solution, and 45 ml. of the borax buffer solution. Mix these in a 500 ml measuring cylinder and dilute to 494 ml. with distilled water. With a pipette add 1 ml of a 1 per cent. aqueous solution of silver nitrate (A.R) and with another pipette 5 ml of a 10 per cent. aqueous solution of pure pyridine. Mix thoroughly.

3. Take the slides from the 20 per cent. silver nitrate and wash for 10 minutes in 3 changes of distilled water.
 4. Place the slides in the impregnating solution (not less than 20 ml. of solution per slide) in covered glass vessels of a kind which can
 - 5
 - 6 place them in the reducer described above for not less than 2 minutes.
 7. Wash in running tap water for 3 minutes: the slides should not lie back to back during this process or traces of reducer will be carried over. Rinse in distilled water. If desired the slides may be left in distilled water.
 8. Tone in 0.2 pc
(The gold chlo
not contain the brown clouds indicative of reduction).
 9. Rinse briefly in distilled water.
 10. Place in 2 per cent. aqueous oxalic acid. The slides remain in this solution for from 3 to 10 minutes and are examined at intervals under the staining microscope. When the axons are thoroughly blue-black the process is stopped; further treatment may result in a diminution of the contrast.
 11. Rinse in distilled water and transfer to a 5 per cent. aqueous solution of "hypo" (sodium thiosulphate) for 5 minutes.
 12. Wash in tap water for 10 minutes, rinse in distilled water, dehydrate, cover.
- Results as in figs. 1 and 5.

Notes. No special precautions are necessary with the glassware, but all pipettes and vessels should be rinsed with distilled water after use and reserved for silver impregnation by this method. Any film of deposited silver on the vessels should be removed. If the silver solution becomes cloudy at any stage in preparation it is contaminated and should be rejected.

The impregnating solution described above consists of an 0.002 per cent. solution of silver nitrate containing 0.1 per cent. of pyridine, and buffered to a pH of 8.4. Variation in the pyridine and silver concentration is permissible and may be advantageous. Thus a heavier, and sometimes more complete, impregnation is obtained by increasing the silver concentration to 0.01 per cent. and the pyridine to 0.2 per cent. In general an increase in the pyridine concentration leads to a more specific but perhaps less complete axon stain, and an increase in the silver concentration gives a heavier deposit.

The method has given good results with material fixed in Bouin, Carnoy and similar mixtures. When these fixatives are used the axon impregnation is sharper than with formol material, but it is a disadvantage that portions of the specimens cannot be reserved for a myelin stain.

progressive process, and the "normal" appearance of a distal nerve trunk will therefore depend upon the time which has elapsed between division and resection of the specimen. There is no gross change in the epineurium and perineurium, though the nerve bundles often become subdivided, so that a distal stump may contain many more bundles than the normal proximal nerve. As the axon and myelin remains are removed (Fig. 9) the Schwann cells multiply mitotically, and the walls of the nerve fibre tube close in, forming a "Schwann tube" whose lumen has a diameter considerably less than that of the normal fibre (compare Figs. 9 and 10, which are to the same scale). The fate of the neurilemma is unknown, for it becomes indistinguishable, but there is a gradual increase and thickening of the collagen fibres in the endoneurium (Figs. 10-12). The great shrinkage of the tubes, is commonly accompanied by an "oedema" of the endoneurium (Figs. 10 and 11).

Regeneration

When the nerve is cut, the axons nearest to the cut surface migrate out into the scar between the stumps. If the axon outgrowths come into contact with the distal Schwann cells they grow down into the distal bundles. Many axon sprouts may enter a single Schwann tube; all of them are at first extremely small in diameter, but as the growing tip passes to the periphery they increase in diameter and correspondingly acquire myelin sheaths. Those fibres which establish a connection with an end-organ increase more rapidly in diameter and eventually become normal or nearly so those which do not eventually atrophy (Sanders and Young, 1946). If none of the fibres establish a distal connection, as when a neuroma is formed, they all remain abnormally small and numerous. The experimental evidence on the factors concerned in regeneration and functional recovery has been reviewed by Young (1942).

The Classification of Nerve Lesions

The reactions of a nerve fibre to injury are such that the lesions which it may suffer fall into three categories, distinguishable clinically and pathologically. These categories have been analysed and

redefined by Seddon (1943) and to them he has given the names "neurotmesis," "axonotmesis" and "neurapraxia." The new classification corresponds approximately to the older "complete division," "lesion in continuity" and "transient block," but it has the advantage that it is based on the reactions of the single fibre, and it is thus possible accurately to define a "mixed lesion" in which, since the fibres have suffered varying damage, the macroscopic appearance of the lesion may be deceptive.

Neurotmesis : The fibre and its endoneurial sheath are divided, and distal to the site of injury it undergoes Wallerian degeneration. If the lesion is extensive the regenerating axons may fail to make contact with the Schwann cells which emigrate from the end of the distal tubes, and the degeneration and paralysis will be unrepaired. But if the nerve stumps lie close together, or if suture is performed, reunion will take place and new fibres will be regenerated in the distal trunk.

Axonotmesis : The fibre is divided, but not its endoneurial sheath ; the neurilemma may or may not be interrupted. Distally Wallerian degeneration takes place, but since the endoneurium is uninterrupted the tube containing the proximal undamaged fibre is continuous with the distal tube with which it was formerly connected. Hence regeneration is rapid and perfect, for the axon outgrowths pass without hindrance into the distal tubes, and the new fibres formed have the same central and peripheral connections as the normal fibres before injury. In regeneration after neurotmesis, on the other hand, however close the stumps there is always considerable confusion at the suture line, resulting in abnormal central-peripheral connections.

Neurapraxia : The nerve fibre fails to conduct impulses past the level of the lesion, but it remains normal distally ; there is no Wallerian degeneration. Neurapraxia due to trauma may be spontaneously repaired, but surgical treatment, such as neurolysis, is sometimes necessary for the restoration of conduction.

Traumatic Nerve Lesions

The examination of minute biopsies has proved to be a valuable aid to diagnosis and prognosis in the treatment of nerve injuries when there is uncertainty as to the degree of damage to the nerve. This technique has been described and illustrated elsewhere (Holmes and Zachary, 1946). Here, therefore, we shall mainly be concerned with the pathological investigation of specimens obtained as a

result of surgical treatment by complete resection of a damaged segment of nerve, carried out as a preliminary to repair by suture or nerve graft. The state of the extreme ends of the excised portion gives a fair representation of the condition of the sutured stumps, and the pathological investigation has as its first objective the prognosis of functional recovery after repair, so far as it is determined by conditions at the suture line.

This prognosis may be so gloomy as to demand a further resection and resuture. And since the condition of the suture line is one of many variables which determine the quality of recovery, it must be investigated in every case if any certainty is to be reached as to the relative significance of all the variables, and every lesion given the optimal treatment.

Handling of Material: If the extent of the lesion is well-defined, the surgeon may need to divide the nerve at two levels only, one above and one below the lesion, and the pathologist will experience no difficulty in identifying the extreme ends. On the other hand it may be necessary to transect the nerve at several levels before nerve tissue apparently suitable for suture is exposed. In this case it is essential that the extreme proximal and distal pieces should be identified by the surgeon and placed in separate labelled vessels of formol-saline. Better still the exploratory sections should be made without dividing the nerve completely; the specimen is then obtained as a string of slices united by epineurial tissue, and the orientation is obvious. To prevent distortion of specimens they should be pressed on to slips of postcard before fixation, and the card with the adherent tissue dropped into the fixative.

For a report on the suture line, slices about 3 mm. thick are cut from the extreme ends of the specimen, and carried through the mordanting for the Weigert myelin stain. Sections are cut transverse to the long axis of the nerve, some stained for myelin, others by the trichrome method.

The sections should of course be cut as near as possible to the extreme ends of the specimen, for even 3 mm. further in the condition of the nerve may be significant. The end cut by the surgeon

each nerve bundle appears by the pathologist is flat.

embedding, and from it the sections are cut.

The Pathological Identification of Nerve Lesions

Gross Appearance : If the specimen presents the familiar appearance of a proximal neuroma separated from the distal trunk, or united with it only by a thin band of scar, then complete neurotmesis without reunion may at once be diagnosed. But if the nerve is more or less continuous the lesion may either be neurotmesis without reunion but with a deceptive continuity of scar tissue, or neurotmesis with reunion, or a mixed lesion, partly neurotmesis, partly axonotmesis. A lesion of pure axonotmesis will not normally be treated by resection.

The Distal Transverse Section : The myelin-stained preparation will provide further evidence. (a) If it contains no myelinated fibres, or fibres so small as to be invisible in Weigert preparations, then the lesion is neurotmesis either without reunion or with reunion of a very poor quality. (b) If a few small scattered myelinated fibres are visible it is probably neurotmesis with poor reunion. (c) If some bundles are full of fibres, while others are empty or only sparsely innervated, there has either been neurotmesis with good reunion at some points only or the lesion is a mixed one, the well-innervated bundles being those which have suffered axonotmesis only (Fig. 7). (d) If all the bundles are well innervated, but with a high proportion of fibres sub-normal in diameter, then the lesion is either neurotmesis with good reunion, as after successful suture, or pure axonotmesis.

The Body of the Lesion : If a full examination is required the remainder of the material, still lying in formol, should be embedded and cut. It may either be treated as a whole and embedded without mordanting, or it may be divided into segments of which some are mordanted for myelin, others embedded directly for an axon stain. For a routine it is usually best to cut longitudinal sections of the whole specimen and stain for axons. If the lesion is suspected to be a mixed one, longitudinal sections should be cut as a series, but it is usually sufficient to preserve only every twentieth or thirtieth section for staining. For a study in the transverse plane the lesion may be divided into some five slices, taking a few sections from one surface of each.

The presence of a neuromatous mass of fibres at the proximal end indicates neurotmesis (Fig. 5); the degree of reunion is indicated by the number and distribution of the axons in the distal bundles. If union is good each tube will contain many regenerating axons (Fig. 1, r). Axonotmesis is ideally characterized by the

PLATE XXVIII

1. Nerve fibres, L.S., axon stain.
 2. Nerve fibres, T.S., myelin stain. n = normal fibres; r = fibres regenerating after axonotmesis; a = axon; m = myelin; en = endoneurium.
 3. Ulnar nerve, normal proximal stump. p = perineurium, e = epineurium.
 4. Fibres in a proximal stump showing endoneurial oedema, T.S., myelin stain.
 5. Neuromatous outgrowth after neurotmesis. L.S., axon stain.
 6. Fibres regenerated after neurotmesis. Myelin stain.
 7. Fibres regenerated after axonotmesis. Myelin stain.
- 1, 2, 4, 6 & 7 all to the same scale as indicated on fig 2.

PLATE XXVIII

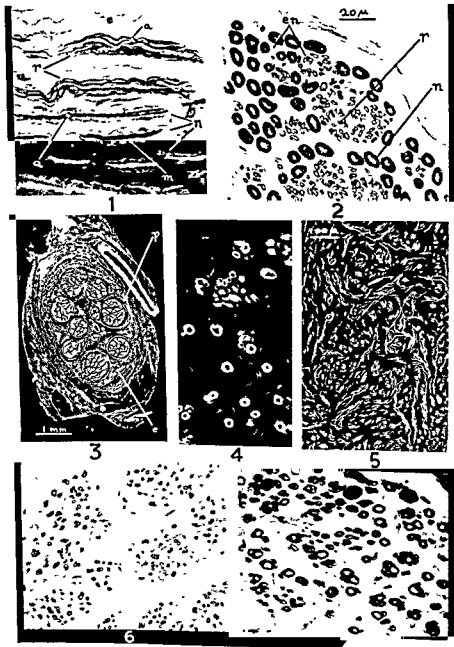


PLATE XXVIII

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 6. Fibres regenerated after neurotmesis Myelin stain.
 7. Fibres regenerated after axonotmesis. Myelin stain.
- 1, 2, 4, 6 & 7 all to the same scale as indicated on fig. 2.

PLATE XXVIII

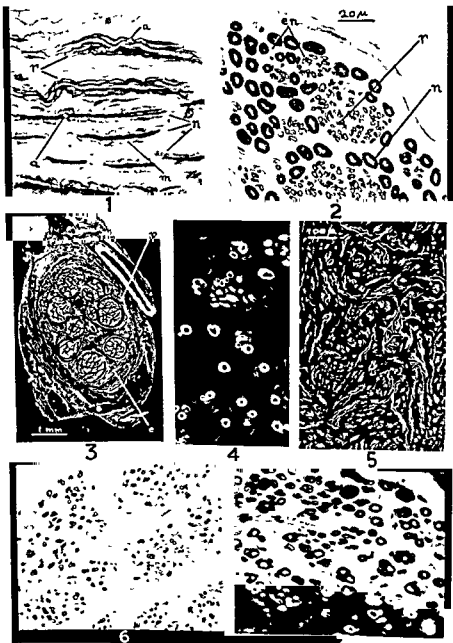


PLATE XXIX

- 8 Distal stump : resection to a " gliomatous " level
9-15 Detail of bundles in distal trunks degenerated for varying times. T S, Masson. All to the same scale as indicated on fig. 9
9 1 month :- St = Schwann tubes ; my = myelin remains ; ma = macrophage ; nl = neurilemma.
10. 3 months ; Sn = Schwann nucleus
11 5 months.
12 13 months
13 & 14 3 months ; abnormal.
15 12 months ; gross collagenisation.

PLATE XXIX



10



11



12



13



14



15

lesions, but the necrosis or obliterative endoneurial fibrosis which may follow severe ischaemia or the accidental injection of chemical substances causes an irreparable separation of the proximal and distal tubes, which amounts to neurotmesis.

Axonotmesis is commonly found in peripheral neuropathies. Since the proximal axon is nourished from the central nervous system, regenerative outgrowths will constantly be put out, and local conditions in the peripheral nerve will determine whether or not full regeneration takes place. Thus when a moderate degree of ischaemia is relieved the degeneration is repaired, as in "Immersion Foot" (Blackwood, 1944); and axonotmesis due to deficiency of vitamins of the B group is repaired when the diet returns to normal (The pathology of vitamin B deficiency neuritis is reviewed by Zimmerman, 1943). If local conditions in the peripheral nerve remain abnormal the regenerative axon outgrowths may show gross abnormalities and fail to become myelinated; the fibre changes under such conditions, as in lead poisoning, are surveyed in detail by de Villaverde (1935).

Neurapraxia, which may not be transient, but enduring, is responsible for many of the characteristic clinical phenomena of neuropathies. A great variety of pathological fibre changes not amounting to axonotmesis has been described in the various forms of "neuritis." Local ("segmental periaxial") myelin degeneration is found in some of the many peripheral nerve disorders that have been attributed to local vascular abnormality (see Fetterman and Spuler, 1940). Similar changes are seen in interstitial hypertrophic neuritis (Sloane, 1939); and in infectious neuronitis (polyradiculoneuritis) (Gilpin *et al.*, 1936). Denny-Brown and Brenner (1944) have studied the pathology of experimental neurapraxia due to pressure, and provide a guide to the methods and results of an investigation of this lesion. A thinning of the myelin sheath has sometimes been reported as a local fibre abnormality. The ratio of myelin sheath thickness to axon diameter is normally constant for fibres of a given total diameter, but on the other hand it departs from the normal during the process of regeneration after axonotmesis or suture. Sanders (1946) has investigated the normal ratio and its changes during regeneration in the rabbit, and his results should be consulted as a guide to the interpretation of abnormalities of the sheath/axon ratio in man.

The nerve fibre lesions are sometimes complicated by abnormality of the sheaths. Endoneurial collagenization has already been discussed as a result of severe ischaemia. In interstitial hyper-

trophic neuritis the nerve trunks are greatly thickened, mainly as a result of the formation of excess concentric lamellae of collagen around the degenerating nerve fibres, producing the characteristic "onion bulbs." The changes in this condition are well illustrated by Wolf *et al.* (1932); they favour the view that the disease is "a primary affection of the Schwann apparatus, characterized by overgrowth and sclerosis."

In some forms of peripheral neuropathy caution is necessary in assuming that the damage is a primary effect of a noxious agent for it may be secondary to interference with the blood supply. Examples of this difficulty are found in the case of cold (Blackwood, 1944, on Immersion Foot) and of pressure (Bentley and Schlapp, 1943; Denny-Brown and Brenner, 1944).

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Normal Appearances of Brain Tissue

Films of normal tissue should be studied before undertaking the diagnosis of pathological material. Portions of cerebral and cerebellar cortex, the white matter and choroid plexus from a fresh necropsy specimen are suggested for this purpose.

The bodies of neurones are recognized by their form, nuclear picture and the presence of Nissl substance (Fig. 1). Myelin sheaths are usually distinguishable by their morphology and faint violet tinge in toluidin-blue preparations. Microglial and oligodendroglial nuclei are densely stained and have thus a pyknotic appearance. The nuclei of astrocytes have a clearer nucleoplasm and hence their chromatin particles are discernible. The cytoplasmic processes of these cells are but faintly seen unless a considerable amount of gliosis is present. The structure of the smaller perforating blood-vessels, and in particular capillaries, is beautifully displayed. The recognition of the granule cells of the cerebellum is important because the unwary may mistake them for tumour cells (e.g. of medulloblastoma) or small lymphocytes. Their disposition in clumps, and the proximity of undoubted Purkinje cells, help in their recognition (Fig. 2). Tufts of the choroid plexus are often withdrawn in exploratory needling of the brain. As a rule their appearance is unmistakable. But difficulty may arise when small groups of the cells, without their supporting stroma, are present in a film. They may then be confused with the endotheliomatous type of meningioma, or with deposits of secondary carcinoma.

Applications of the Technique

The principal use of the method is perhaps in the diagnosis of tumours explored with the brain-needle. In the Nuffield Department of Surgery the needle-biopsy is frequently combined with ventriculography, and the films then made are examined while the X-ray investigation is in progress. With the information gained from these different angles the surgeon decides upon the next step. The wet-film has an advantage over the frozen section in that every fragment of tissue removed can be examined with the microscope provided that it is suitably spread upon the slide. The technique may also be used for the examination of any cellular material withdrawn in the tapping of cysts during craniotomy. It is also used when, during an operation, the surgeon encounters tissue concerning which further information is desired. Thus a tumour of the cere-

bellum in a young child may be a medulloblastoma or an astrocytoma. If the former, the procedure may be conservative; if the latter, a radical removal will probably be attempted.

DIAGNOSTIC FEATURES

I. Tumours

The principal cellular features of the tumours most commonly requiring examination by this technique are as follows:

1. **Meningioma** (Figs. 3 and 4): The cells have distinct borders and are of polygonal epithelioid form, or spindle-shaped. The epithelioid areas appear as sheets with irregularly branching tongues at the periphery, recalling the appearance of fibroblasts in tissue culture. The presence of whorls of cells or of psammoma bodies is diagnostic. The nuclei are oval with a nucleoplasm that has less affinity for stains than in the gliomas. They contain a few small chromatin nodes and one or two nucleoli. Vacuolar spaces within the nuclei are a common and quite characteristic feature (Fig. 4). Mitotic figures are extremely rare.

2. **Medulloblastoma** (Figs. 5 and 6): The tissue forms a film with the greatest facility and microscopically is usually homogeneous, being composed of small spheroidal cells with scanty indefinite cytoplasm and a nucleus that stains so deeply as to obscure the chromatin. Mitotic figures and multinucleated cells are often numerous. Differentiating examples may show cells with definite uni- or bipolar processes. Exceptionally rosettes of Wright's type (1912) are present (Fig. 6). The example illustrated was later proved to contain many undoubted neuroblasts.

3. **Ependymoma** (Figs. 7 and 8): Films are easily prepared, but are usually less homogeneous than in (2) owing to the architecture of the tumour. Thus pseudo-rosettes of cells about blood-vessels predominate in many examples. In these each cell has a single tapering process directed towards the vessel. In films a papillary effect is thus produced (Fig. 7). Between the perivascular zones there is generally a loose-textured arrangement of polygonal epithelioid cells (Fig. 8) sometimes enclosing spaces in a manner suggestive of true rosettes (Flexner, 1891), though well-defined rosettes of this type have not yet been encountered in films of ependymomas. The nuclei are ovoid or spheroidal, even in size and contain numerous small nodes of chromatin in a lightly staining nucleoplasm. Mitotic figures are rare.

4. **Oligodendroglioma** (Fig. 9): Films are prepared with great ease, the tumour tissue often being of gelatinous consistency. Characteristically the oligodendroglial cells are in the degenerative phase known as "acute swelling" and this is clearly visible in the film. Each nucleus occupies a clear space representing the highly vacuolated cytoplasm, and adjacent cells are separated by the tumour matrix. This is sometimes of a mucinous character, giving a lilac metachromatic stain with toluidin blue. The nuclei are spheroidal and are apt to vary both in size and in chromatin content. Mitotic figures are often present. Sometimes the tissue is composed of undegenerated cells, especially if obtained from the edge of the tumour. These, like the normal oligodendroglial cells, have a scanty cytoplasm prolonged into a few exceedingly delicate processes. The cells tend to be arranged in small clumps, or may be linked by their processes to form short strings. Calcospherites may be present.

5. **Astrocytoma** (Fig. 10): The more fibrillary of these tumours are difficult to spread and have a characteristic tough rubbery texture. Diagnosis is obvious in examples composed of stellate cells with long, interlacing fibrillary processes. In pilocytic examples the cells are elongated and bipolar, but are otherwise similar. In the gemistocytic type, composed of plump astrocytes, the cells are swollen and more closely packed, and bear relatively scanty but coarse processes. The cytoplasm is homogeneous and glassy while the nucleus is eccentric. Mitoses are absent. Their presence, in what would otherwise appear to be an astrocytoma, is suggestive of early anaplasia, and a fuller examination of the tumour may reveal more extensive changes in the direction of spongioblastoma multiforme. Calcospherites are occasionally present. Cuffing of the vessels with lymphocytes may be encountered, but, in distinction to the gliosis about chronic inflammatory foci, these cells are confined to the vessel walls. In diffusely infiltrating examples of astrocytoma the nervous elements, especially bodies of neurones, may be included in the tissue.

6. **Spongioblastoma multiforme** (Figs. 11 and 12): These tumours are easily filmed. In a large proportion of them the microscopical appearances are unequivocal. The immense variety in the shape and size of the cells ranging from fully differentiated astrocytes to small polygonal cells, the variation in the chromatin content of the nuclei, the uni- and multinucleate giant-cells, the presence of typical and atypical mitoses, and the great thickening of blood-vessels through endothelial proliferation all contribute to

the picture. In areas where astroblastic forms predominate a papillary arrangement of the cells about the vessels may be seen (Fig. 11). Necrotic tissue is often withdrawn by the needle and, in instances where almost the whole of the tumour is altered in this way, it may be impossible to obtain any further details. In such circumstances it may be presumed that the tumour is either a spongioblastoma multiforme or secondary carcinoma when there is supporting clinical history of the rapid development of symptoms in an adult subject.

7. Choroid Plexus Papilloma (Fig. 13): The appearances of this tumour so closely resemble those of the normal choroid plexus that the chief difficulty lies in distinguishing between normal and neoplastic tissue. In the papilloma there is, however, a tendency for the epithelium to be columnar rather than cubical.

8. Pituitary adenoma (Fig. 14): Few tumours in this class have been examined by the wet-film technique because the diagnosis is seldom required at operation. Those examined have been composed of polygonal cells of variable size, with well-defined borders and a cytoplasm that sometimes contains fine eosinophil granules in Mann's stain, *i.e.* transitional acidophil cells. The nuclei are often eccentric and contain one or two rather conspicuous nucleoli and a few chromatin nodes in a lightly staining nucleoplasm.

9. Secondary Carcinoma (Fig. 15): Manifestly the appearances in this group as a whole are too varied to be treated in detail. The sharp transition from the groups of tumour cells to the nervous tissue in which they are embedded is, when the latter tissue also has been withdrawn, a characteristic feature in comparison with spongioblastoma multiforme—the only tumour with which secondary carcinoma is likely to be confused. The outlines of the cells are sharp. Moreover the nuclei in secondary carcinoma are usually distinguished by the conspicuous nucleoli and lightly stained nucleoplasm. Mitotic figures and areas of necrosis are often abundant in these tumours.

10. Plasmacytoma (Fig. 16): Two examples of this tumour have been examined. The plasmacytoid nature of the cells is well displayed in toluidin-blue preparations, and the characteristic metachromatic staining with Unna-Pappenheim's method is obtainable in films.

II. Non-neoplastic Conditions

1. Chronic Focal Inflammations: Films of tissue from the neighbourhood of a chronic abscess may show the following features:

fibrillary gliosis with multiplication of astrocytes, cuffing of vessels with large mononuclear cells, lymphocytes and variable numbers of polymorphonuclear leucocytes, infiltration of the adjacent nervous tissue with similar cells and compound granular corpuscles. Similar appearances may be found about granulomata; the presence of typical Langhans' giant-cells is diagnostic of tuberculoma.

2. *Torula* (*Cryptococcus hominis*) though rarely encountered is readily diagnosed in wet-films. The capsule is well preserved and stains an intense lilac colour with toluidin blue owing to its mucinous content. Reduction of the time in this stain permits a clearer distinction between the capsule and the centrally placed organism. Small lymphocytes and numerous macrophages are associated with the organisms.

PLATE XXX

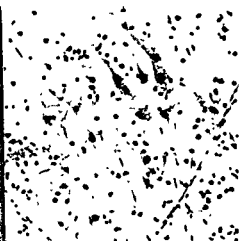
- 1 Normal cerebral cortex showing pyramidal cells, glial cells and vessels. Toluidin blue $\times 120$.
- 2 Normal cerebellar cortex with granule cells (left lower); 2 Purkinje cells near centre. Toluidin blue. $\times 200$.
- 3 Meningioma. Two whorls of cells near one corner. Toluidin blue $\times 210$.
4. Meningioma showing vacuolar nuclei. Mann's eosin-methyl blue $\times 385$.
5. Medulloblastoma. Toluidin blue $\times 320$.
6. Medulloblastoma. Showing rosettes. Mann's eosin-methyl blue $\times 240$.

3. **Cerebral thrombo-phlebitis:** Films show areas of extravasated red corpuscles mixed with variable numbers of polymorphonuclear leucocytes. The sheaths of the vessels may be infiltrated with similar leucocytes. There is oedema and degeneration of the nervous elements, and mobilization of compound granular corpuscles (Fig. 17).

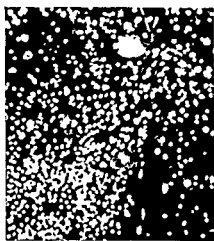
4. **Calcification of Blood-vessels** (Fig. 18): This is well displayed in films. While the significance of this change cannot often be assessed it is sometimes found in the neighbourhood of slowly growing gliomas, such as astrocytoma.

Pitfalls in Diagnosis

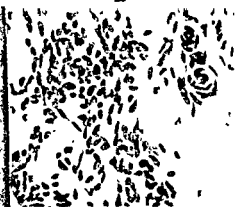
Although a correct diagnosis can be returned in a large percentage of cases, errors have been made especially when minute fragments only have been available for examination. The following examples may serve as a warning to others.



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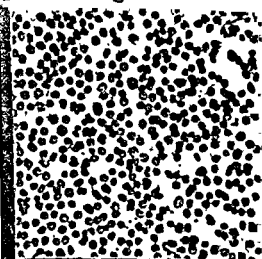
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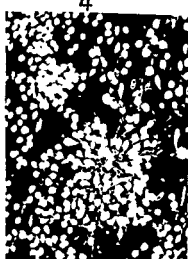
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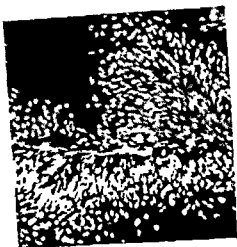
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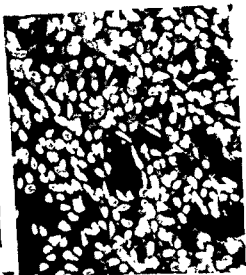
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PLATE XXXI

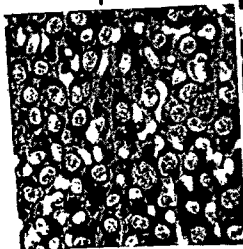
- 7 Ependymoma characteristic orientation of cells to blood-vessels. Toluidin blue $\times 145$
- 8 Ependymoma : area of cells without vascular orientation. Toluidin blue $\times 230$
- 9 Oligodendroglioma : cells in phase of "acute swelling." Mann's eosin-methyl blue $\times 320$
- 10 Fibrillary astrocytoma Toluidin blue $\times 140$.
11. Spongioblastoma multiforme showing papillary arrangement of tumour cells about vessels, and a giant-cell. Toluidin blue $\times 150$
- 12 Spongioblastoma multiforme . polymorphism of cells. Toluidin blue $\times 240$.



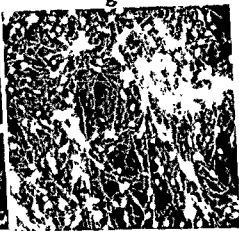
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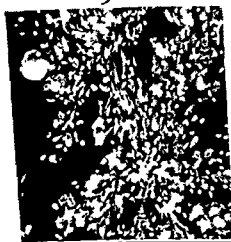
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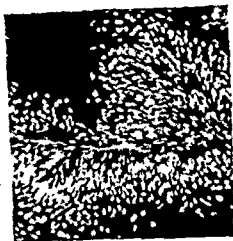


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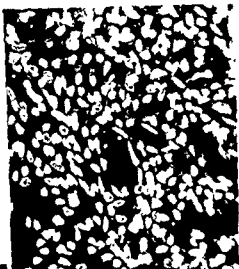
PLATE XXXI

7. Ependymoma . characteristic orientation of cells to blood-vessels. Toluidin blue $\times 145$.
8. Ependymoma area of cells without vascular orientation Toluidin blue. $\times 230$.
9. Oligodendroglioma . cells in phase of "acute swelling." Mann's eosin-methyl blue. $\times 320$.
10. Fibrillary astrocytoma Toluidin blue. $\times 140$.
11. Spongioblastoma multiforme showing papillary arrangement of tumour cells about vessels, and a giant-cell. Toluidin blue. $\times 150$
12. Spongioblastoma multiforme . polymorphism of cells Toluidin blue $\times 240$.

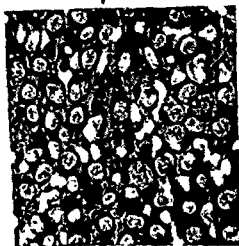
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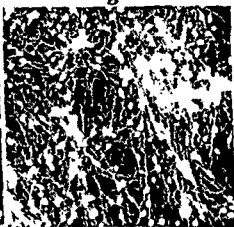
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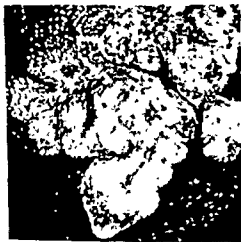
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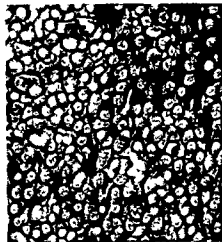
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PLATE XXXII

- 13 Papilloma of chroid plexus Toluidin blue. $\times 90$
14. Pituitary adenoma: chromophobe type with transitional phil cells. Mann's eosin-methyl blue. $\times 240$.
- 15 Secondary carcinoma (primary in lung). Toluidin blue
- 16 Plasmacytoma of frontal bone. Toluidin blue. $\times 240$.
- 17 Compound granular corpuscles. Toluidin blue. $\times 400$
- 18 Capillaries showing focal calcification. Toluidin blue. \times



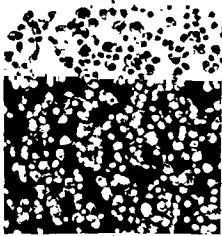
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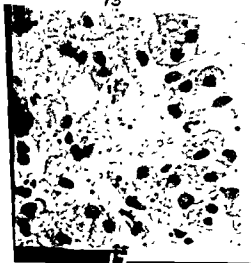
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CHAPTER XL

THE CLINICAL AND POSTMORTEM PATHOLOGY OF ENCEPHALITIS

THE term "encephalitis" in its broadest interpretation covers all forms of inflammatory disease of the brain; it includes those caused by pyogenic bacteria or by higher forms of vegetable life such as the pathogenic cryptococci and also infection of the brain by certain protozoal and metazoal parasites. In its usual connotation, however, it refers mainly to those diseases of the brain which are known to be, or are suspected of being, due to viruses. While it is rarely possible on pathological grounds alone to determine which virus is the cause of a particular case of encephalitis, the combination of clinical and epidemiological evidence with the pathological findings may sometimes give adequate grounds for an exact diagnosis. More often this can only be established by transmitting the disease to laboratory animals or by studying the immunity reactions in the serum of patients who have recovered from the disease. During the past twenty years there have been several large epidemics of encephalitis in the United States, the U.S.S.R. and Japan from which viruses have been transmitted and studied. With the exception of the outbreak of post-vaccinial encephalomyelitis none has occurred in this country where only sporadic cases of encephalitis have been seen, and attempts to transmit virus from these have been, almost without exception, unsuccessful. The evidence accumulated by studies of the epidemics and by laboratory experiments, especially in the United States, has made it possible to formulate a broad histological classification of the types of encephalitis so far encountered.

The cause of several of these histological types is not yet known. Other types are known to be produced by several different viruses; but all the viruses causing one type show a broad similarity in the method by which they enter and attack the brain. A histological classification is therefore the only one possible at present; such a classification cannot be final, but it is hoped that it may be found to be closely correlated to any future classification based on aetiology and pathogenesis.

CLINICAL PATHOLOGY OF SUSPECTED
CASES OF ENCEPHALITIS

The diagnosis of encephalitis during life, especially in the absence of an epidemic, depends more on elimination than on finding specific changes in the cerebro-spinal fluid and blood. In some forms of encephalitis and in some epidemics the changes in the cerebro-spinal fluid, taken along with the clinical symptomatology, are sufficiently characteristic to give strong grounds for a diagnosis of encephalitis. But many diseases, some primarily affecting the brain, others chiefly affecting other organs of the body, may simulate encephalitis clinically. It may indeed often be impossible, on clinical grounds alone, to make a definite diagnosis until the stage of recovery has set in.

1. Cerebro-spinal Fluid: Lumbar puncture should be done with all the precautions used when cerebral tumour is suspected. A fine needle should be used, the pressure should be recorded and the amount of fluid removed should be little more than will permit of the necessary examinations being made. These include cell count, bacteriological examination, estimation of total protein, glucose and chlorides, and the gold or other colloidal reaction.

Pressure: Some rise of pressure of cerebro-spinal fluid is usually present in the acute stages of encephalitis. This depends on the degree of congestion of cerebral capillaries and may attain between 200 and 300 mm. of water.

Appearance: The fluid is usually clear and does not tend to form a coagulum. When the cell count is high there may be a slight turbidity.

In
lur
acute types of encephalitis such as Eastern equine encephalo-myelitis and in the St. Louis epidemic, turbidity and the formation of a coagulum have been more common. Some degree of xanthochromia, usually along with the presence of red cells, is not uncommon in the more haemorrhagic types of the disease.

Cell Count: This varies enormously from 0 to 2,000 per c mm. or more, depending on the type and severity of the case. Most commonly the cell count lies between 10 and 100 per c mm. and consists chiefly of lymphocytes. But a varying proportion of polymorphonuclears has been found in some epidemics and they may be in a majority in certain of the more acute types, especially during early stages, e.g. in polio-encephalitis and in acute haemorrhagic leuco-encephalitis. In the Eastern type of equine encephalo-myelitis the cell count is high (200-2,000 per c mm.) in the early stages and the majority of the cells are polymorphonuclear.

In the neurological complications of mumps, and in benign lymphocytic choriomeningitis, cell counts between 100 and 1,000 per cmm are common, but lymphocytes always predominate and may be the only type of cell present. In some cases of post-infectious encephalomyelitis with a comparatively low cell count a considerable proportion of the cells appear to be monocytes. While the highest cell counts are often encountered in the early stages of the disease, in some epidemics high cell counts have not been found until the disease has been established for several days.

Protein: As a general rule the increase of protein in encephalomyelitis bears little relationship to the cell count. Thus in the epidemics of encephalitis lethargica a "cell-protein dissociation" (high cell count with normal protein) was often found and was considered a cardinal sign of the disease. This dissociation has often been found in other forms of encephalitis during the early stages. On the other hand in post-infectious encephalitis the protein often rises along with the cell count, and in the more acute forms with very high cell counts there is always a considerable rise in the protein.

Colloidal Reactions: The colloidal gold, benzoin and other similar reactions have been found to vary greatly. In most cases a weak curve in the luetic zone is obtained, but occasionally there has been a strong curve in the paretic zone. This was noticed first during the epidemics of lethargic encephalitis where such strong reactions did not appear to be correlated either to the cell count or to the clinical aspects of the disease. They appear however to be more common in forms of encephalitis affecting the grey matter primarily than in those which have a more diffuse incidence on the tissues of the brain.

In some cases of Dawson's type of encephalitis with intranuclear inclusion bodies and of a subacute sclerosing encephalitis a strong paretic type of colloidal gold reaction has been the only abnormality discovered in the fluid.

Chlorides. Estimation of the chlorides in the cerebro-spinal fluid is of value only as an aid in eliminating other possible diagnoses since in uncomplicated cases of encephalitis the chlorides always remain within normal limits (700-750 mg. per 100 ml). Vomiting, diarrhoea, pneumonia or, especially in children, a sharp rise of temperature leading to great thirst, may cause lowering of the chlorides to a less or greater degree. Meningism in children which may mimic meningitis or encephalitis clinically, is often associated with a limited fall in the chlorides to between 650 and 680 mg. as the only abnormality in the fluid.

The common fall in the chlorides in tuberculosis and other forms of generalized meningitis is well known, and serves as a valuable pointer in the differential diagnosis. But localized bacterial infections of the brain or the meninges (including subdural empyemas) do not usually lead to any fall in the chlorides, and therefore no help in the differentiation of such infections from encephalitis will be given by chloride

estimations. On the other hand the presence of a high lymphocyte count in the fluid (100 or more) with normal chlorides is in favour of a viral rather than a bacterial cause for the symptoms. In rare cases of coma due to nephritis, the chlorides have been found greatly raised to between 900 and 1,000 mg. This has been shown by Linder to be due to the associated acidosis and appears to occur only within a few days of the fatal termination. In such cases examination of the cerebrospinal fluid may act as a pointer to the renal disease.

Glucose: As with the chlorides, no alteration in the glucose content of the fluid is usually present in encephalitis, but in some cases it may rise along with a corresponding rise in the blood sugar; more important, no fall in the glucose (in the fresh fluid) occurs even in the presence of very high cell counts; there is a striking distinction between the almost constant fall of the glucose in tuberculous meningitis to below 30 mg., and its equally constant normal level in fluids from cases of encephalitis with a similar cell count. In the same way fluids from encephalitic cases with a high polymorphonuclear cell count may be readily distinguished from those of meningococcal or other forms of bacterial meningitis by the presence in them of normal or high percentages of glucose.

Another value of glucose estimation is to eliminate cases of hypoglycaemic coma, since glucose virtually disappears from the cerebrospinal fluid in these cases. It seems probable that certain of the cases of acute hepatitis, wrongly diagnosed as encephalitis, pass into coma and die chiefly as a result of hypoglycaemia.

Wassermann Reaction. The meningo- and neuro-recidives of syphilis may be wrongly diagnosed as encephalitis, and the Wassermann reaction in the fluid may in such cases be the only point of differential diagnosis.

Blood Count. In some epidemics of encephalitis (e.g. St. Louis epidemic, Eastern and Western equine encephalo-myelitis) the blood shows a polymorphonuclear leucocytosis during the first few days. In cases due to the Eastern strain of the virus of equine encephalo-myelitis, the leucocytes have risen to 65,000 per c.mm. A leucocytosis in the later stages of the disease is more likely to be due to pulmonary complications.

Postmortem Technique in Suspected Cases of Encephalitis

The treatment of the brain at the postmortem examination should follow a definite procedure designed to ensure that the microscopical examination is not rendered impossible by random slicing or by failure to obtain good fixation. Slicing of the fresh brain is necessary for early fixation which is essential for the finer histological study; pieces from selected areas should be retained either frozen or in buffered glycerine for virus studies. Transfusion of fixative through the carotids prevents any study of the virus and is therefore contra-indicated.

The following method of slicing is recommended :

The brain should be removed with as long a stalk to the medulla as possible. For this purpose the tentorium should be cut round its attachments to the skull almost to the middle line, and the nerve roots and vertebral vessels cut across before the medulla or upper cervical cord is transected. A long pointed pair of scissors is better than a knife for this purpose.

The spinal cord should also be removed entire. The upper cervical segments, which are frequently left in the body at postmortem examinations, are especially important in cases of suspected encephalitis.

After examination of the surface of the brain, including the main basal vessels, the mid-brain is transected horizontally. Further horizontal sections are then made through the middle of the pons and at the junction of the pons with the medulla. These may be carried right through the cerebellum, but it is better first to detach the cerebellum from the brain stem by oblique vertical sections across its peduncles, a short distance away from the brain stem, and then to make sagittal vertical sections through the vermis and lateral lobes. These give a better plane for microscopical study of the cerebellar foliae. The cerebral hemisphere are then turned base upwards and coronal sections are made with a long knife through the entire organ, starting at the tip of the temporal poles and at intervals of an inch or rather more behind this to near the occipital poles. Pieces of the frontal and occipital poles about 10 cm. in antero-posterior extent are not too large to fix well. Too much slicing of the brain at this stage is to be avoided.

In selecting pieces for virus studies there should be as little disfigurement as possible of the larger pieces, as after fixation these should be placed together in their original position for the selection of pieces for histological examination. The areas of the brain likely to be most useful for virus studies are :

- (1) One half of the midbrain.
- (2) The hippocampal gyrus along with the fascia dentata at about its middle third.
- (3) The basal ganglia.
- (4) The precentral gyrus.

Australian and American workers have obtained more success in transmission of virus to animals by keeping the tissue frozen than by preserving it in buffered glycerine.

The best fixative for general purposes is 15 per cent. to 20 per cent. formalin (6-7 per cent. formaldehyde) in 1 per cent. sodium chloride solution. With reasonably strong commercial formalin 15 per cent. is adequate, but weaker percentages are rarely satisfactory unless very large amounts of fluid are used (*e.g.* 5 or 6 litres). The quantity of formalin used is perhaps of more importance than the percentage since the 1,100-1,400 g. of tissue in the brain is capable of absorbing a large

amount of formalin during the process of fixation. As a general rule 600 ml. of formalin (35-40 per cent. formaldehyde) made up to 4 litres with saline is sufficient for the fixation of a sliced-up brain.

For finer histological studies small pieces may be placed directly in a solution containing 5 per cent. mercuric chloride and 10 per cent. formalin in water but this fixative does not allow of silver impregnation methods.

The ordinary earthenware "brain-pots" measuring about 20 cm. in diameter by 10 cm. deep are too small for the fixation of a whole brain even after slicing, but the pieces can be distributed in two or three of these pots if nothing larger is available. Enamelled bins 20 cm. in diameter by 17 cm. deep are more satisfactory. If not sliced before fixation the brain should be suspended in the bin by a string passed under the basilar artery and tied to the handles.

The pieces of brain commonly taken for microscopical study in general diseases of the brain are :

- (1) *Cortex*, prefrontal, Rolandic (pre- and post-central), calcarine, hippocampal (just behind the uncus). These should be cut as far as possible at right angles to the lines of the sulci and the run of the convolutions.
- (2) *Basal ganglia* at three antero-posterior levels to include (a) head of caudate nucleus; (b) greatest width of body of lenticular nucleus; (c) greatest width of thalamus.
- (3) *Midbrain*, with special attention to the substantia nigra and nucleus ruber.
- (4) *Hypothalamic nuclei* which lie in the tuber cinereum and the anterior part of the wall of the third ventricle. This area is preferably sectioned in the coronal plane, sections being kept from different levels for about 1.5 cm. backwards from the tuber cinereum.
- (5) *Pons, medulla* (upper limit and below inferior olives).
- (6) *Cerebellum*, vermis and lateral lobes.
- (7) *Spinal cord* (a) upper cervical segments (C 1 to C 4); (b) cervical enlargement; (c) mid-thoracic level; (d) lumbo-sacral enlargements.
- (8) One or more *dorsal root ganglia* and *Gasserian ganglion* (this is readily exposed by tearing the dura mater from the base of the skull).

Morbid Anatomy of Encephalitis

As a rule the brain in cases of encephalitis shows no definite macroscopical abnormality on external examination. The pial vessels are often congested and the grey matter may be more red than normal, but this capillary congestion may be no more than is found in patients dying of respiratory failure.

In encephalitis due to a pan-tropic virus (Type II) there is often some degree of infiltration of the basal meninges or cloudiness of the cerebrospinal fluid. The softening of the white matter can sometimes be recognized in post-infectious encephalitis (Type III). In acute haemorrhagic leuco-encephalitis (Type IV) the petechial haemorrhages and the oedema of the white matter of the cerebral hemispheres make it possible to arrive at a diagnosis from the gross appearance alone.

Histology

Staining Methods. Although special methods are valuable for the study of some of the finer points in the histology of encephalitis, only routine tissue stains are needed for the diagnosis of its type and main features. A good alum haematoxylin and eosin stain gives especially valuable information both as regards the inflammatory reaction and the neuronal degeneration. Cell stains such as phloxin-methylene blue or Giemsa or toluidin blue in one of its modifications are also valuable.

Stains for myelin, especially Scharlach R on frozen sections and Loyez haematoxylin on celloidin or paraffin sections are useful but, rarely necessary for diagnosis. Silver staining for microglia, astrocytes or neurones, belongs rather to the special study of the disease process rather than to routine diagnosis.

HISTOLOGICAL CLASSIFICATION

TYPE I. Virus Encephalitis affecting the Grey Matter primarily and almost exclusively

When the virus has been transmitted and studied it has behaved as a strictly neurotropic virus, infection by the blood stream being difficult or impossible. The degeneration of nerve cells may be concentrated in certain groups or nuclei (e.g. substantia nigra in encephalitis lethargica, the pyramidal layer of the hippocampus in rabies, the anterior horn cells of the spinal cord and motor nuclei of the brain stem in poliomyelitis). Inclusion bodies either intra-cytoplasmic (rabies) or intra-nuclear (poliomyelitis, Dawson's subacute form of encephalitis) may be found in the nerve cells. Degeneration of nerve fibres may also occur in the most severely affected areas of grey matter.

Forms: (1) Rabies; (2) Encephalitis lethargica and sporadic forms of unknown aetiology; (3) Poliomyelitis and polio-encephalitis of the brain stem; (4) Subacute encephalitis with intra-nuclear type A inclusion bodies (Dawson).

TYPE II. Encephalitis of both Grey and White Matter caused by Virus of Pan-tropic Type

Transmissible to animals by the blood stream and in some forms transmitted to man from an animal reservoir by the bites of blood-sucking insects. It is characterized by focal accumulations of small cells, chiefly of microglial and oligodendroglial or lymphocytic types (inflammatory nodules). These are found scattered in a widespread manner through the nervous system, being usually most numerous in the brain stem. They occur both in the grey and white matter, and may or may not be related to small vessels; central necrosis may occur in some of them. Degeneration of nerve cells may be confined to the vicinity of the inflammatory nodules or may be more widespread. A varying amount of perivascular and meningeal infiltration is present. Inclusion bodies have not been seen in the brains of human cases, but occur in the kidneys in cases of St. Louis encephalitis and in animals infected with equine encephalomyelitis.

Forms: (1) St. Louis form; (2) Japanese B. encephalitis; (3) Eastern, Western and Venezuelan forms of equine encephalomyelitis; (4) Russian spring-summer tick-borne encephalitis; and (5) Herpes virus and louping-ill virus encephalitis in man; (6) Subacute sclerosing encephalitis.¹

TYPE III. Post-infectious Encephalitis (Acute Perivascular Myelinoclasia)

A disease characterized by narrow zones of demyelination, with partial destruction of axons, round small venules throughout the nervous system. The disease may be concentrated on the brain-stem and basal ganglia, the cerebral hemispheres or the spinal cord. The walls of the ventricles and the meningeal surface of the spinal cord may be affected. There is microglial and histiocytic infiltration of the demyelinated zones but perivascular and meningeal lymphocytic infiltrations are usually slight and may be absent. Degeneration of nerve cells is minimal. The pathogenesis is unknown, but the disease tends to follow an exanthematous or other infectious disease at an interval of several days after its onset.

Forms: Following (1) vaccination; (2) variola; (3) measles; (4) influenza; (5) varicella; (6) mumps; (7) apparently occurring spontaneously.

¹ The nosological position of subacute sclerosing encephalitis is not clear as no virus studies on this form have yet been made. Its histological characters, however, are those of Type II encephalitis in subacute form.

PLATE XXXIII

1. Acute encephalitis of the grey-matter (Type I) (cause unknown). A cortical vein is surrounded by a cuff of lymphocytes. There is a diffuse increase in size and number of microglial cells. The nerve cells are so degenerated as to be almost unrecognisable as such. (Iron Haematoxylin & van Gieson) $\times 200$.
2. Subacute encephalitis with inclusion bodies (Type II). (Case of Dr. Russell Brain.) Group of anterior horn cells in the cervical enlargement of the spinal cord, one of which is degenerated and contains an intra-nuclear inclusion body. (Haematoxylin-eosin) $\times 230$.
3. The same case. Anterior horn cell in cervical enlargement of spinal cord. A large nerve cell with a laterally placed nucleus shows an intranuclear inclusion body and intra-cytoplasmic granules staining purple. There is much neuroglial and microglial increase in the neighbouring tissues (Mallory's phosphotungstic acid haematoxylin). $\times 200$.
4. Post-morbillar encephalomyelitis (Type III). A vein in the white matter of the brain surrounded by a zone of demyelination and excess of microglial and histocytic cells. (Iron Haematoxylin—van Gieson). $\times 8$.
5. Acute haemorrhagic leucoencephalitis (Type IV). Corpus callosum showing perivascular zones of myelin destruction and numerous petechial haemorrhages (black). (Loyez myelin stain.) $\times 15$.
6. Acute haemorrhagic leucoencephalitis (same case). Polymorpho-nuclear and mononuclear exudate round vessel in neighbourhood of corpus callosum $\times 260$.

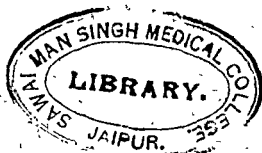
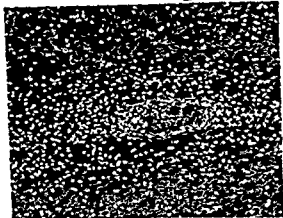


PLATE XXXIII



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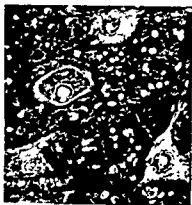
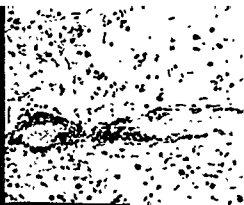


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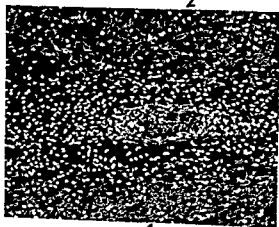
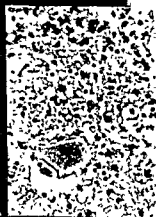
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6. Acute haemorrhagic leucoencephalitis (same case). Polymorpho-nuclear and mononuclear exudate round vessel in neighbourhood of corpus callosum. $\times 260$.

PLATE XXXIII



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6

degeneration. Some degree of astrocyte reaction is also seen in the perivascular zones of demyelination in post-infectious encephalitis especially in cases which have survived for more than a week. In the foci of degeneration and softening seen in acute haemorrhagic leucoencephalitis and in the Eastern type of equine encephalomyelitis there is acute degeneration of astrocytes (clasmatodendrosis). In this condition the nucleus may be large, lobed or reniform and stain palely, or the chromatin may form dark granules under the nuclear membrane. The cell body is ragged and the processes cannot be stained.

J. G. GREENFIELD.

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Type I.

Rabies.

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(1941) 55.

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SILBER, L A, & SOLOVIEV, V. D. *Amer. Review of Soviet Med* (1946) Spec Supplement

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VAN BOGAERT, L. *J. Neur. Neurosurg. & Psych.* 8 (1945) 101.

Type III.

*Post-infectious Encephalomyelitis**Variola*MARSDEN, J. P. & HURST, E. W. *Brain* 55. (1932) 181.*Influenza.*GREENFIELD, J. G. *Journ. Path & Bact* 33 (1930) 453

Type IV.

*Acute Haemorrhagic Leucoencephalitis*HURST, E. W. *Med Journ of Austral* 2 (1941) 1.HENSON, R. A. & RUSSELL, D. S. *Journ. Path & Bact.* 54 (1942) 227.

Type V.

*Mumps.*DONOHUE, W. L. *Journ Ped.* 19 (1943) 42

Other Forms.

*Toxoplasmic*WOLF, A., COWEN, D. & PAIGE, B. H. *Amer Journ Path* 15 (1939) 657.STEINER, G. & KAUMF, D. H. *J Neuropath & Exper. Neur.* 3. (1944) 36*Virus Pneumonia.*INGLEBY, H. *Arch Path* 37. (1944) 359*Rocky Mountain Spotted Fever.*HASSIN, G. B. *Arch Neur & Psych.* 44. (1940) 1290SCHEINKER, I. M. *Arch Path* 35 (1943) 583*Typhus Fever.*SPIELMEYER, W. *Histopathologie des Nervensystems* (1922) Springer, Berlin.GROSS, W. *Virchow's Arch f Path Anat* 242. (1923) 452

compression; the centre of a scirrhus carcinoma may show no neoplastic cells. In sarcomata the edge may be more or less differentiated than other parts, so that for accurate classification pieces from different parts and of differing appearance must be examined. In simple neoplasms there may be compression at the edge, and the surgical habit of incising close to an apparently benign tumour often produces intravital trauma of the tissues at the edge; the edge must be taken however for possible invasion of the capsule, as well as portions from the varying appearances in the tumour itself. In a specimen of hypernephroma the renal vein should be examined; of a tuberculous kidney, the ureter; and of a pathological testis, the spermatic cord. Lymph nodes are often difficult to find in axillary fat but within an hour or two of removal it is still usually possible to feel them; the nodule should be gripped in the left hand and a very sharp knife used to cut down and through it. On this freshly cut face one can tell if the nodule be fatty or lymphoid. Single nodes removed for microscopy should be cut into slices by the sharpest fine razor before fixation and two fixatives should be used (formol-sublimate and Zenker, Bouin or Susa).

The proper selection of tissues from postmortem likewise depends on the knowledge of the pathologist. It is important to remember that if no gross lesion be discovered during the routine autopsy then the left ventricular myocardium and the kidney should both be examined microscopically, and the gastric contents kept for the chemist. The neophyte may well follow the old hand in unashamedly storing tissues, surgical or postmortem, until the microscopic appearances have been seen.

Finally it may be recalled that rough handling by the surgeon produces gross change in the tissues, and that neither this damage nor the alterations due to autolysis can be rectified by any method known to the pathologist; both add to the difficulties of diagnosis and either may make it impossible.

4. Fixation

The ideal fixative for routine material is formol-sublimate (Lendrum, 1941a, sometimes called formol-corrosive). This is an apparently stable mixture of 9 parts of saturated aqueous mercuric chloride (5.6 per cent.) with 1 part of commercial formalin.¹ It is a much more rapid fixative than formalin alone (which as Mallory always maintained is a poor fixative by itself for tissues to be

¹ These are the proportions suggested by Dr. Carleton in his first edition, 1926

embedded in paraffin) and yet it is much less distorting or destructive than plain mercuric chloride, and a month's immersion does no harm. An advantage of its rapid coagulation is that a larger piece of tissue may be put in the fixative than would reasonably be handled; within 8-18 hours the piece may be trimmed to workable size by cutting away unnecessary bits, and the face desired for microscopy is by this time well enough coagulated to maintain its shape during the subsequent treatments. It pays to have at least twenty times more fixative than tissue, and to support the tissue on cotton wool. For small fragments, such as curettings, bottles of fixative (lids must not be aluminium) are supplied to the clinicians; the whole technique of the small biopsy is fully described elsewhere (Lendrum, 1943).

5. Fixatives for Special Purposes

i. Calcified Tissues: After 24 hours' fixation in formol-sublimate, tissues are transferred directly to Da Castro's fluid (chloral hydrate 20 g., nitric acid 20 ml., 95 per cent. spirit 250 ml., water 250 ml) in an open dish; the fluid is changed daily until the tissue is soft. The tissue is then washed for 24 hours and taken to paraffin as usual. For lesser amounts of calcium and sometimes even for heavy bone, a modified Ebner's solution proves most satisfactory. The tissue is again transferred directly from fixative to the decalcifying solution (6 per cent. mercuric chloride in 15 per cent. sodium chloride, with 1 per cent. hydrochloric acid, a further 1 per cent. of acid is added each day) and dehydration is begun (omit wash) as soon as a sufficient depth of tissue is decalcified (see also section 7, p. 448).

ii. Fat is generally studied in frozen sections for diagnostic purposes, and is well preserved in formol-sublimate. The block should be washed briefly before cutting; the mercury discolours the razor but seems to do little harm otherwise. The sections are treated with iodine and "hypo" before staining. If it is intended from the beginning to stain for fat then part of the tissue could be fixed in formalin (see 9, ii, p. 449).

iii. Glycogen is in part retained by formalin-sublimate; there is insufficient coagulation with formalin alone to entrap the water-soluble glycogen. If a secondary growth is suspected as being from an hypernephroma a snippet should be fixed in Bouin, Carnoy or 95 per cent. spirit with 10 per cent. formalin.

iv. Haemopoietic System: If one wishes to use Romanowsky stains, fresh tissues are fixed in Zenker's fluid (6-8 hours),

and postmortem tissues in the Zenker stock solution without the addition of acetic acid. This is also suitable for tissues from cases of malaria (Lendrum, 1944a). If the blood is beginning to break down, as in extravasations, thrombi or postmortem material the fixative that causes least further lysis is formalin; an hour or two in this may save the more fragile erythrocytes and further fixation can then be done in formol-sublimate or Zenker (without acetic). The precipitate formed by the interaction of blood pigments and formalin can be simply removed from sections by Barrett's (1944b) ingenious use of alcoholic picric acid.

v. **Museum Specimens**: Fixation should be in large amounts of Kaiserling's or Jores's solution; formalin 10 per cent. is nearly as good but mercurial fixatives must not be used.

vi. **Nervous System**: see 10, xvi, p. 457.

6. The Progression to Paraffin

For the diagnostic general pathologist paraffin is still the ideal embedding material and for routine purposes there are two main routes thereto. One is rapid because certain types of small loose tissues are apparently suited to this; the other is slower since it gives on average good histology with the general run of tissues. The fetish of rapid reporting has probably caused more bad diagnoses than it ever accelerated the proper treatment of the patient, and the rare case of urgency can be dealt with specially. Curettings from uterus or sinuses (nasal or inflammatory) and biopsies from other internal epithelia are handled as described (Lendrum, 1943). There is no need to wash tissues fixed in formol-sublimate or to remove the mercuric chloride until the section is on the slide (iodine and hypo).

The general run of surgical tissues is transferred on the first day after operation from fixative to 70 per cent. spirit in the morning or at midday, and in the evening to 95 per cent. spirit containing 6 per cent. phenol; on the second day to 100 per cent. ethanol (or equally good, "absolute spirit," which is industrial methylated spirit 74 degrees overproof¹) with one change, total duration

¹ Alcohols other than ethanol are now widely used in histology, and for research material we prefer butanol (*n*-butyl, Lendrum, 1941b), but for routine purposes we use industrial methylated spirit in the two grades, "66 overproof," the methylated spirit of about 95 per cent alcohol content commonly used, and "74 overproof." This latter is about as water-free as absolute ethanol, is much cheaper, and its content of methanol apparently does no harm to the tissues.

6-8 hours, followed by two hours in equal parts of absolute spirit and chloroform, and then overnight in chloroform. On the third day the tissues are shifted to the second chloroform bottle in the morning, to paraffin in the oven at midday, changed to second paraffin in the evening, and blocked in filtered paraffin on the morning of the fourth day. The best paraffin (M.P. 52° C. suits the climate of Glasgow) is a wise economy but even it may be improved (made more microcrystalline) by the addition of 5-10 per cent of beeswax. Small circular metal tins, *sandbaths* (from bakers' sundriesmen) are very suitable for blocking; we use them of diameter 2.5, 5 and 10 cm., and depth 2.5 cm. Certain tissues need special handling and if this be appreciated there is no reason why the majority of tissues should not be entrusted to an automatic changing machine as now available in America. If a tissue be particularly tough, it pays to increase the time of dehydration and to follow the chloroform by treatment with benzol¹; the time in the oven may also need prolongation even to 24 hours. At the other extreme, lymph glands and similar cellular tissues such as liver must on no account be left too long in the oven; four to eight hours is ample for these and overcooking will shrink and crack such tissues irretrievably. The difficulty of diagnosis of the lymphadenopathies demands the most scrupulous care in their preparation. - The use of the vacuum for paraffin impregnation shortens the time and is an ideal method; it is to be hoped that in the near future there will be an oven of large size with a thermostat and an exhausting system, both of which will be rapidly self-regulating to a temperature and negative pressure that can be set as wished by the operator.

In emergency an electric bulb (preferably carbon filament) can be used to melt the top layer of a dish of paraffin and so impregnate tissue. Tissue is safely stored only in paraffin.

The type of containers suggested by Cook and Satterfield (1941-2) for dehydration and clearing would probably suit the moderate sized laboratory.

Celloidin embedding: see 10, xvi, p. 458.

¹ Chloroform is expensive but following dehydration by ethanol it is the least hardening of the volatile paraffin solvents; the use of benzol after chloroform seems to have only slight hardening effect, whereas its use directly after ethanol is rapidly hardening and only suitable for small tissues that can be quickly cleared.

and boil for 5 minutes. When cool it is ready for use. If not needed at once, the boiling is omitted since ripening occurs at room temperature to an optimum at about three months; after six weeks' ripening, 2½ minutes' boiling will bring the staining qualities up to maximum. With some brands it pays to double the content of haematoxylin. If the tap water is acid, Scott's Tap Water Substitute (KHCO_3 2 g., MgSO_4 20 g., distilled water 1 litre) blues the haematoxylin safely and quickly and needs only short washing thereafter. Eosin is a unique dye, and at its best gives a polychromasia unapproached by any other cytoplasmic stain. For routine staining it is used as a 1 per cent. aqueous solution, containing 2 per cent. calcium chloride (see Conn and Holmes, 1928). If the ethanol, during dehydration, leaches too much of the eosin away from the collagen for the taste of the observer, he may use instead 1 part of Lugol's iodine to 9 parts of aqueous eosin; this stains very rapidly (too long action of the iodine affects the nuclear staining) and is distinctly less susceptible to the ethanol. H. & E. provides the basis of most pathologists' experience and is adequate for the diagnosis of most of the routine tissues, but certain conditions cannot be fully classified merely on the H. & E. section, while the difficult case may be unexpectedly clarified if a set of other methods be tried, particularly the next three.

II. Van Gieson: With the modern mounting medium (see section 11) this stain is reasonably permanent (better than H. & E.) and is of established value in the diagnosis of the fibroma-neurofibroma group

Stain celestin blue 10–20 minutes in jar, (less if fresh), rinse tap water.

Filter on Mayer's haemalum 5–10 minutes, rinse tap water.

Scott's tap water substitute 1–2 minutes, wash tap water.

Stain van Gieson (1 per cent. aqueous acid fuchsin 15 ml., sat. aqueous picric acid 50 ml., dist. water 50 ml.) 5 minutes, *do not wash*.

Dehydrate rapidly with absolute spirit, clear and mount.

Preparation of celestin blue: Allow 2.5 g. iron alum to dissolve overnight at room temperature in 50 ml. distilled water: to this add 0.25 g. of celestin blue R (C.I. 900) and boil for 3 minutes: filter when cool into a staining jar and add 7 ml. glycerol.

This solution maintains its efficiency for 6–12 months, while the haemalum is the routine solution. The combined dyes give a satisfactory nuclear stain much more easily and consistently than does

van Gieson solution is of proportions that give results with Revector fuchsin (magenta acid); dye from other makers might need different proportions (see McFarlane, 1944, for discussion on picro-fuchsin).

III. Mallory's Method and Its Modifications: The value of

dye, lies in the fact that the collagen can be strongly stained and differently coloured from the cytoplasmic elements. On the whole the methods based on this principle are used more with postmortem than with surgical tissues, but with both they provide constant help, and the general technique should be mastered in every laboratory (see recommended texts or Foot, 1933, and Lillie, 1938).

The original method had a virtue possessed by few of its endless progeny; it stained fibrin red and blood yellow. If this distinction be wished for diagnostic purposes it will be found that the picro-Mallory methods (Lendrum and McFarlane, 1940, McFarlane, 1944 with valuable discussion) allow the worker to obtain the colours originally described but rarely achieved since then; in place of the cytoplasmic dyes mentioned there—acid fuchsin, ponceau or Biebrich scarlet—with some tissues it is worth using a mixture of 3 parts 1 per cent. aqueous lissamine fast red BS (I.C.I.) with 1 part of 1 per cent. aqueous picric acid.

If the distinction between fibrin and blood is less desired than strong nuclear staining or clear delimitation

Example:

- i. Stain celestin blue (see 10, ii) 5 minutes in jar (less if fresh), rinse tap water.
- ii. Filter on Whatman's No. 541.
- iii.
- iv. 5 minutes.
- v. Immerse in 1 per cent. aqueous phosphomolybdic acid (put jar in 56° C. oven), 5 minutes.
- vi. Rinse 1 per cent. aqueous acetic acid.
- vii. Stain in jar of 1 per cent. soluble blue (C.I. 706) in 1 per cent. aqueous acetic acid, 5-15 minutes.
- viii. Rinse 1 per cent. acetic, dehydrate, clear and mount.

The crystal ponceau¹ (step iv above) has the advantage over acid fuchsin of being rapidly removed from the collagen and of giving a pleasant transparent cytoplasmic staining; Biebrich scarlet may be used similarly (Lillie, 1940) and is almost as good. If the collagen in the section is fine or scanty, it is sometimes worth replacing the blue

¹ Suggestion from Mr. D. McFarlane.

(stage vii) by 1 per cent. naphthalene black 12B200 (I.C.I.) in 1 per cent. aqueous acetic acid. When a prominent staining of muscle, particularly fine fibrils, is desired then in step iv in place of the ponceau we use 1 per cent. lissamine fast red BS (I.C.I.) in 1 per cent. aqueous acetic acid, and instead of the blue in step vii use 1.5 per cent. tartrazine NS (I.C.I.) in 1.5 per cent. aqueous acetic acid; sections are transferred directly from the tartrazine jar to a jar of 65 per cent. ethanol (water removes the tartrazine too quickly) and after a very brief rinse are dehydrated by dropping on absolute spirit. This method gives a very striking demonstration of the finer muscular tissue, such as that in the pulmonary trabeculae or the basal muscular layer of mammary epithelium. Lissamine red is also excellent for acidophil granules, although in sections of pituitary we prefer to follow it, at stage vii, with a green stain, edicol pea green H (I.C.I.) 1 per cent. in 1 per cent. aqueous acetic acid, or 2 per cent. fast green FCF in 2 per cent. acetic acid. For specialized and important applications of the Mallory principle to staining of the pituitary, see McLetchie (1944).

In many cases the following simple method may prove adequate:

Stain in nuclear lissamine red 3 minutes.

Rinse very briefly in water.

Picric differentiator 5 minutes.

Rinse briefly in water.

Stain picro-edicol 5 minutes (see below) or 1 per cent. edicol pea green or soluble blue (as given above) 10 minutes.

Rinse, dehydrate, clear and mount.

Nuclear Lissamine Red: Dissolve 0.3 g. lissamine fast red BS (I.C.I.) and 10 g. aluminium sulphate in 100 ml. distilled water. Boil gently for 10 minutes, restore the evaporated water, and when cool add 7-25 drops of 7 per cent. aqueous ferric chloride (the larger quantity makes the stain more specifically nuclear, and gives less cytoplasmic staining).

Picric Differentiator: 2 g phosphotungstic acid, 2 g phosphomolybdic acid, saturated aqueous picric acid 70 ml., 95 per cent methylated spirit 30 ml.

Picro Edicol: Mix equal parts of 2 per cent. aqueous edicol pea green H (I.C.I.) and saturated aqueous picric acid in a staining jar and add acetic acid to 2 per cent.

iv. Reticulin: This rapid method, first worked out by Drs D. C. Caldwell and I. Rannie in this laboratory owes its original ideas mainly to Gordon and Sweets (1936) and to Gömöri (1937 and 1939). See also McGregor and Hill (1943). It may lack some of the delicacy of the longer methods, but it is relatively foolproof and does not remove the section from the slide even in the absence of gelatin adhesive (see section 8).

Flood the section with 0.25 per cent. potassium permanganate containing 0.3 per cent. sulphuric acid for 10 minutes. Bleach in 1 per

cent. oxalic acid. Wash tap water 5 minutes. Mordant 15-20 minutes in jar of 2.5 per cent. iron alum (dissolved without heat). Wash tap water 10 minutes. (If final staining proves diffuse or beaded increase washing time on the restaining up to 20 minutes.) Silver solution, room temperature 3 seconds. [Silver solution: 10 ml. of 20 per cent. silver nitrate (dist. water) in 100 ml. cylinder. Add 0.4 ml. of 40 per cent. sodium hydroxide. Add strong ammonia (about 1 ml.) by drops until there is left the merest speck of precipitate. Make up to 50 ml. with dist. water and put in a staining jar, coated with dark paper and bound with strong adhesive tape. The solution keeps for several months, although it may according to Smith (1913) become violently explosive if kept even for 24 hours. If the staining time becomes too long, fresh silver solution is prepared; the jar is not cleaned before refilling.] Rinse in tap water,¹ and reduce in 10 per cent. formalin, 30-60 seconds. Rinse and observe. If understained, remordant with alum and restain as above. If overstained, put through iodine and hypo (as for removing sublimate deposit), rinse, mordant again and give shorter time in silver. If the only fault be slight background deposit (this may be got with freshly made silver solutions during the first 5 or 6 days), then tone in 0.2 per cent. gold chloride for 3 minutes, toning is mildly reducing but is not essential. Rinse in tap water. Fix in 5 per cent. aqueous hypo 2-5 minutes. Wash well and counterstain. Counterstaining after reticulin is often difficult and sometimes it is essential to obtain a good nuclear stain, especially in neoplasms of the reticuloendothelial system (see 10, v). Reticulin, including the basement membrane of the renal glomerulus, is well shown by the method of Lendrum, Carson and Penny (1915); this seems to show the fine fibrils as clearly as does the silver method given above, and if preceded by celestin blue and haemalum staining of the nuclei gives a good general picture. The formalin vapour treatment is essential. The iodine treatment is better omitted. A reticulin method is a necessary part of the investigation of the lymphadenopathies neoplasms of the reticuloendothelial system and those tumours that seem to lie between carcinoma and sarcoma.

v. Red Nuclear Stains: With human material there are not easy to obtain with any certainty. For Gram's method fast purple carmalum (see Muir and Ritchie, or Castleman) is generally excellent, after reticulin staining, however, it may fail to give enough definition but 10 minutes in carmalum followed by a three and then 1 minute in 0.5 per cent. aqueous neutral red, provides much a detail distinctly better than is obtained by either dye alone. This sequential neutral red is also good after elastic staining or the picrocarmalum method for iron. With some tissues it may be worth using one of the following: Careful

¹ Glasgow tap water is almost always necessary at this stage

² In other districts distilled water

safranin : melt 0.5 g. phenol in a dry flask by rotating under the hot tap. To this add and mix well 0.1 g. safranin (C.I. No. 841); the sludge is then dissolved in 50 ml. starch-dextrine solution (grind 0.25 g. starch to fine powder, then grind in 0.25 g. dextrine, add 50 ml. dist. water gradually with grinding; heat to 80° C. and filter when cool). The solution is ready for use and maintains its activity for months in a staining jar. After 10 minutes staining the section is differentiated either in spirit or in 15 per cent. formalin (6 per cent. formaldehyde) in which the dye is both fixed and differentiated.

Marshall Red : This dye introduced by Cannon (1941) has proved of some value with human tissue if made up as follows : Dissolve 0.5 g. Marshall red (B.D.H.) in 50 ml. starch-dextrine solution (as given above) to which 0.1 g. sodium metaborate has been added ; heat to 70° C for 5 minutes. When cool this is ready for use and is used in a staining jar (10-30 minutes).

Gallego's Method (Langeron, p. 555) : Water 10 ml, Ziehl's carbol-fuchsin 5 drops, glacial acetic, 1 drop. Stain 5 minutes, wash and then differentiate and fix for 5 minutes in water 10 ml., formalin 2 drops, glacial acetic, 1 drop.

vi. Romanowsky methods : The interaction of the fluorescein group, eosin, erythrosin and phloxin with the thiazin blue dyes still remains a difficult method for the worker with human tissues, as is proved by the number of modifications published. For the diagnostic histologist their use seems indicated very rarely in surgical material, while fine diagnosis of blood diseases can and should be made before death. The myeloid cells alter rapidly after death and the tinctorial shades used in the smears of the expert haematologist to classify the stages of the erythron cannot be obtained with any certainty in sections. Recent Romanowsky methods for sections have been published by

(1938) merits further attention.

vii. Elastic Tissue : The following type of Weigert solution is due mainly to French (1929), and works well and consistently. 200 ml. dist. water in flask, dissolve in order : 1 g. crystal violet, 0.5 g. commercial dextrine, 4 g. resorcin, 1 g. basic fuchsin. Boil and add 25 ml. boiling 29 per cent. ferric chloride ; boil for 3 minutes, cool rapidly and filter. Dry filter paper with precipitate in oven (56° C.). Add dry precipitate and paper to 110 ml. 95 per cent. spirit and leave at room temperature for 24 hours. Bring to boil on hot plate and boil for 1 hour. Cool and make up to 150 ml. with 95 per cent. spirit. Take 50 ml. of this stock (keep remainder in stoppered bottle) and add 0.5 g. phenol and 2 ml. hydrochloric acid and filter into a staining jar. This keeps for several months. The usual staining time is 60-90 minutes.

although previous thorough treatment by permanganate and oxalic (Mallory bleach, see 10, iv) seems to accelerate and improve the staining. Differentiate in spirit. Counterstaining may be by light haemalum followed by a rinse with tartrazine NS (I.C.I.) saturated solution in cellosolve (Lendrum, 1939), or by any red nuclear stain, or by the Masson type of Mallory, particularly the lissamine red and tartrazine method (section 10, iii).

viii. Striations in Muscle: The demonstration of these in a sarcoma is occasionally demanded (although seldom urgently) and for routine purposes there is little doubt that the ideal method is a not too fully ripened phosphotungstic acid haematoxylin—"phostox." The stain can be made from haematoxylin crystals (Masson (1923) gives haematoxylin 0.1 g., phosphotungstic acid 0.2 g.—not 2.0 g. as in Langeron and in Mallory—water 80 ml and 0.2 ml. hydrogen peroxide as ripener) and is ripe in about 12 days at room temperature. Sections are stuck to slides by the gelatin method, and mordanted for 24 hours at room temperature in Lugol's iodine; they are then given the Mallory bleach (permanganate and oxalic, see 10, iv), washed and stained in "phostox" for 24 hours at room temperature. Do not wash; dehydrate rapidly with absolute spirit and clear. If the collagen is stained the rich orange-reds of the classical method, the solution is too ripe for the scheme as given, and one must then reduce the times of mordanting and staining, and prolong the treatment with alcohol. If the ideal colour balance is caught, striated fibres are much more easily found than in sections stained by iron haematoxylin; indeed this latter for all its beauty (when good) has no place in our usual routine diagnostic methods, since the celestin blue-haemalum combination proves more consistent as a routine for van Gieson and Masson stains. Those interested in iron-haematoxylin should consult Ide-Rozas (1935) and the valuable survey and original work of Cole (1943) on haematoxylin stains.

ix. Eosinophils in tissue are shown up clearly by carbolchromotrope (Lendrum 1944b). Melt 1 g. phenol crystals in flask by rotating the flask under the hot tap; to this add and mix " " trope 2R (C.I. 20) then ordinary haemalum, rinse and dehydrate. Blood films.¹

Carbol may also be used with

x. Plasma Cells: Neither Unna-Pappenheim nor the malachite green—acridine red method of Hitchcock and Ehrlich (1930, and see Kerr and Lendrum, 1935) gives consistently good results. Better pyronins are reported from America (Conn, p. 141) but the writer has no experience of them. Recently we have found that two hours' treatment of the section with Hollande's fixative (neutral copper

¹ Personal communication from Mr. Vance Carlisle, Rhodesia.

acetate 2.5 g., picric acid 4 g., formalin 10 ml., glacial acetic acid 1.5 ml., water 100 ml.) improves staining by the malachite green-acridine red mixture; after a brief rinse the section is stained for 10 minutes, dehydrated directly by cellosolve and so to xylol; from xylol the section is differentiated in tartrazine-cellosolve-xylol mixture (see 10, xvii), rinsed in pure cellosolve and back to xylol. This is a controllable method and with well-fixed tissue gives a picture of great delicacy; but a more easily applied and more vigorous stain that would reveal plasma cells and at the same time delineate clearly the remainder of the tissues, is particularly wanted for uterine curettings, since the plasma cell is the best index of chronic inflammation therein. Romanowsky methods (see 10, vi) are sometimes very satisfactory for plasma cells.

xi. Amyloid: Methyl violet 1 per cent. aqueous gives the most vivid demonstration of this material. An intensification of the metachromatic reaction is brought about by primary mordanting in 1 per cent. aqueous stannic chloride for 15 minutes. Oxalic acid 2 per cent. is a rather better differentiator than the usual acetic acid. If the section be then treated with 10 per cent. aqueous sodium chloride for 10 minutes, washed for 20 minutes in water, and mounted in Karo (see section 11), it is fairly permanent. For balsam mounts, stain by haemalum followed by 3 minutes in saturated aqueous lithium carbonate, then 1 per cent. aqueous congo red for 15 minutes; differentiate in 50 ml. jar of 95 per cent. spirit plus 2 drops saturated alcoholic solution fast green FCF, dehydrate, clear and mount. In the liver, Mallory's method, as he suggested in 1900, shows amyloid clearly.

xii. Glycogen: Best's stain suffers from fairly rapid deterioration of the solution, thus control material should always be available (blocks of hypernephroma) and stained in parallel. A final rinse with tartrazine in cellosolve (see 10, vii) seems to increase the specificity of the stain as used in the standard way. Material fixed in formol-sublimate usually does not come off the slide but sections otherwise fixed should be stuck on slides by Masson's gelatin method. A recent critical study is given by Bensley (1939) on the demonstration of glycogen and the use of a Bauer-Feulgen method. We have obtained good results with a Feulgen solution containing no hydrochloric acid: 0.2 per cent. basic fuchsin (not base) in water was decolorized by the addition of 0.6 per cent. $K_2S_2O_8$ and 1 per cent. $NaHSO_3$. Mordant section overnight in 1 per cent. chromic acid, wash 5 minutes, stain Feulgen solution 60 minutes, rinse three times ($1\frac{1}{2}$ minutes each) in 11 per cent. $NaHSO_3$, wash 10 minutes, haemalum as usual, followed by tartrazine in cellosolve.

xiii. Iron: The microscopic demonstration of iron is well reviewed by Gömöri (1936). Here, 1 part of 4 per cent. aqueous potassium ferrocyanide is mixed with 3 parts 4 per cent. hydrochloric acid in a

test-tube, heated to 60° C. and then flooded on the slide; staining is complete in 5 minutes. A red nuclear stain is then used. The dinitrosoresorcinol method of Humphrey (1935) has the disadvantage of needing ammonium sulphide. For the relation of iron and calcium, see Cameron (1930).

xiv. Melanin: If a tumour is suspected to be melanoma, stain with haemalum alone as eosin covers slight melanin colouring. The silver methods have not proved very helpful in the doubtful routine case. Melanin is bleached in sections (affixed by gelatin) by treatment with dilute hypochlorite (Milton).

xv. Mucin: Southgate's (1927) mucicarmine is still an excellent method; it should be noted that it may be a fortnight after preparation before the stain is ripe. Some commercial solutions work well but a periodic control should be done on a section of intestinal mucosa. The principal value of the stain is in those case of carcinoma of stomach with unicellular diffuse infiltration, and of course in secondary carcinomatous nodules. Sections are first stained by haemalum, blued in Scott's tap water substitute, rinsed very briefly in water, stained in mucicarmine (diluted 1:6 of water) for 20 minutes, returned to tap water substitute, washed, rinsed with tartrazine in cellosolve to give a delicate yellow stain to the cytoplasmic elements, rinsed with alcohol¹ and cleared.

Recently it has been found that mucin stains very distinctly with celestin blue mordant section in 5 per cent. aqueous aluminium chloride (or potassium alum) 1½ hours, wash 5 minutes, treat with tap water substitute 5 minutes, wash 10 minutes, stain 20-30 minutes in 0.1 per cent celestin blue (1 part of stock 1 per cent. celestin blue in alcohol to 9 parts water), rinse water (if desired stain briefly with tartrazine in cellosolve), dehydrate, clear and mount. This gives a finer metachromasia than the toluidin blue method, even in the modified form in which after staining with 1 per cent. aqueous toluidin blue, the section is differentiated in 1:1,000 aqueous acetic acid and then dehydrated rapidly with acetone (Lillie, 1929). A study of the classical methods has been given by Sylvén (1938a and b).

xvi. Nervous System: For this, the compendious little book by Dr. Dorothy Russell is indispensable, and has the merit, so rare in histological texts, of appreciating the particular needs of the general pathologist suddenly faced by a diagnostic problem in this specialty. One other method, that of La Manna (1937) justifies mention as a consistent and simple way of producing the Weigert picture in cord or brain from human postmortem material.

Fix in 10 per cent unneutralized formalin for 4 days or longer. Transfer directly to an aqueous solution of 0.5 per cent. potassium

¹ Cellosolve is miscible with both water and xylol. Tartrazine is insoluble in xylol, slightly soluble in alcohol and very soluble in water.

bichromate with 4 per cent. zinc chloride (an excellent mordant); put the dish in the 56° C. oven for 24 hours. Wash 6 hours, dehydrate and embed in paraffin. Cut thick sections 25-30 μ and stick to slides by gelatin adhesive (essential). Bring sections to water, mordant 30 per cent. ferric chloride 1 hour, rinse briefly in water and immerse in 5 per cent. ferric chloride for 5-10 seconds. Rinse briefly in water and stain in haematoxylin for 1 hour (0.5 g. haematoxylin dissolved in 10 ml. ethanol and diluted to 100 ml. with 0.5 per cent. aqueous acetic acid. This can be used at once and retains its activity in a staining jar for several weeks). Blue in half saturated lithium carbonate. If necessary, differentiate in 0.2 per cent. aqueous hydrochloric acid (this is slow but safe, even if left overnight); reblue and counterstain if desired—we use Gallego's nuclear stain (see 10, v) followed by a rinse with tritrazine in cellosolve. Lillie (1944) has recently given a controlled study of Weigert myelin methods for paraffin sections.

Celloidin, which is a high viscosity nitrocellulose may be replaced by Necoloidin (I.C.I.). The cheaper low viscosity nitrocelluloses are being advocated in America; these have a wider range of solubility (Jordan, 1940) and the solutions are of lower viscosity and so more penetrant, but the dry substance is harder and much less plastic than a high viscosity nitrocellulose. It is possible that the addition of non-volatile solvents (plasticizers) may make low viscosity nitrocellulose a usable substitute. Thus far our only success with this modification has been in the double embedding technique (Lendrum, 1941b); this has definite value with cellular and soft tissues, but is rather slow.

xvii. Organisms: For Gram positive organisms in sections, the clearest and cleanest results are given by Kirkpatrick's method (Muir and Ritchie, p 119); nuclear staining by carmalum is followed¹ by crystal violet, after a wash the section is treated for 2 minutes by 5 per cent. aqueous sodium chloride (in place of the usual iodine). The

more strongly than the other methods. . . .
 tions of thionin from depositing crystals but a starch-dextrine solution is fairly stable: dissolve 0.5 g. thionin in 1 g. warm phenol; dissolve this sludge in 100 ml. starch-dextrine solution (see carbol-safranin, 10, v) and then add 2 ml. of 10 per cent. sodium metaborate. Stain for $\frac{1}{2}$ -1 hour, wash well, bring to xylol and mount or transfer to a jar containing 5 ml. saturated tartrazine in cellosolve, 30 ml. cellosolve and 15 ml. xylol; differentiate to taste, then drop on cellosolve to remove excess yellow staining, and finally clear in xylol. In tissues with much blood, crystallization of the thionin seems commoner but

¹ For demonstration purposes it pays to stain for 5 min in 0.5 per cent. aqueous aurantia between the carmalum and the violet (see Conn for oxalate violet formula).

treatment for 2 hours in saturated alcoholic picric acid followed by 30 minutes wash before staining, seems to remove the substance (? formalin deposit) that favours crystallization. The celestin blue method given for mucin (10, xv) stains organisms in sections; with 10 minutes staining the capsules of pneumococci are shown up meta-chromatically but longer staining as is necessary for the bacterial bodies produces a less obvious capsular staining.

Tubercle bacilli in sections stain poorly, if at all, even with a good carbol-fuchsin (Muir and Ritchie, p. 116) if the sections be stuck on the slide by the gelatin method; this has been traced to the effect of the formalin vapour (confirming the observations of Fielding, 1934). Following Fielding we have tried various alkalized

... exposed to the hot formalin vapour, carbol-fuchsin seems to reveal more organisms than do the alkaline solutions. Room temperature staining for 24 hours with carbol-fuchsin does not seem to reveal as many organisms as the routine method:—heat to steaming on the slide and allow the stain to act during 10 minutes' cooling, wash, differentiate with 20 per cent. sulphuric acid, wash and stain lightly with haemalum followed by a light counterstaining with tartrazine cellosolve (see 10, vii).

The demonstration of certain inclusion bodies of phloxinophil type is satisfactorily achieved by modification of the writer's original phloxin tartrazine method. Haemalum staining of the nuclei is followed by a general overall staining with phloxin and subsequent prolonged treatment with a saturated solution of tartrazine in cellosolve; this removes the phloxin from different tissues in turn and is of value in that certain inclusions show a remarkable affinity for and retention of the phloxin, remaining red when all other elements in the section have been rendered yellow. (Lendrum, submitted *J. Path. and Bact.* 1946)

11. Clearing and Mounting

The clearing of sections before mounting often causes cracking in fragile tissues such as liver or brain; this is lessened if the transition from alcohol to xylol be done by stages (Dr. Janet Niven). In place of Canada balsam we use a synthetic resin, a polystyrene soluble in xylol to give a colourless solution (Distrene 80, British Resin Products Ltd., Epsom, Surrey). This has maintained the colour of sections distinctly better than Canada balsam (Kirkpatrick and Lendrum, 1939, 1941), but its use demands that mounting be done from xylol that is absolutely free from paraffin wax. Unlike balsam it does not tolerate alcohol. To remove a mounted cover-slip, immerse in trichlorethylene or (less rapid) benzol.

B.P.S. mountant : Dissolve 10 g. Distrene 80 in a mixture of 5 ml. dibutylphthalate and 35 ml. xylol.

As a mountant from water, Karo corn syrup¹ (Patrick, 1936) has proved very satisfactory. The medium is made by diluting Karo with one or two parts of water and adding a crystal of thymol; this keeps well in the refrigerator, and the mount dries with a fairly firm edge.

12. Marking

For writing on slides, bottles or tins, Friar's balsam and soluble blue (or other anilin dye) diluted with alcohol as needed proves satisfactory if a fine steel nib is used.

Charting of a field on a slide is best done by the Maltwood type of finder. If a metal frame be fitted along the top and right-hand short side of the finder, and the bottom left-hand corner of the finder-slide be cut off, the left thumb can fix the slide (carrying the section) while the right hand slips the finder-slide over the other so that the metal frame is in contact with the top and right-hand short side of the section-slide. It would be a help if the manufacturers made an oblong numbered grid in place of the usual square.

13. Museum Specimens

The first essential is the difficult one of envisaging the final specimen. Fixation should be in large amounts of Kaiserling's or Jores's solution; formalin 10 per cent. is nearly as good but mercurial fixatives must not be used. It is important to use plenty of cotton-wool and to pack out any cavities with wool soaked in fixative. Thick layers of wet wool help to flatten a surface and this may be reinforced by a glass plate; thin walled open structures should be pinned on a layer of wet wool on a board. Penetration by these fixatives is poor, so that if it is intended to show a section of an organ, the face should be immediately exposed by a single cut heel to toe, with the 25 cm. brain knife (to avoid wave marks) and immediately fixed. Fix for 7 to 28 days. Further details may be found in the standard texts.

14. Research

Research on technical methods is possible even in the routine laboratory thanks to the excess of tissue generally available; thus one portion put through by the standard method serves to provide the diagnosis and acts as control to those undergoing the method being tested. There is little doubt that in a routine laboratory attempts to improve a technique or to master a new one have a most invigorating effect on all the staff, and indirectly raise the

¹ Corn Products Co., Ltd, Bush House, London, W.C. 2.

quality of the general routine preparations. For those without intrinsic original ideas the technical journals provide a steady flow of histological methods which if not fit for adoption may well suggest adaptations of current practices; a method excellent for the pituitary of the mouse can be quite unsuitable for human pituitary but it may have a value for some other human tissue and so the process of trial and selection goes on to build up the morbid anatomist's technique. If the end result be a finer section then it is certain that the diagnosis will be nearer the truth.

Finally it must be said that what is good in this chapter is the product of the persistent and scrupulous attention to detail, over many years, of Sir Robert Muir and those privileged to be his medical or technical assistants. The writer accepts full responsibility, however, for what is said and what is omitted, and would merely ask that if there be any help herein to the busy routine worker, it should be credited to these. To this he would add his own tribute of gratitude to many of these, present and past, for teaching, collaboration and friendship.

ALAN C. LENDRUM.

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